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Systems biology approaches in metabolic engineering: harnessing the potential of proteomics to understand and engineer metabolism

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Abstract

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The genomic revolution has powered the development of numerous genetic sequencing tools, which have contributed to unveiling the genomes of different life forms. This boom has impacted the development of techniques to efficiently manipulate organisms through metabolic engineering and synthetic biology, and of systems biology tools for the analysis of cellular systems via functional genomics. Among functional genomics, proteomics provides valuable insights on the entire set of proteins, which are the main components of the physiological pathways in the cell. In this study, three different systems were analyzed via proteomic profiling to understand metabolism and identify targets for engineering. First, the toxic response of *Escherichia coli* to short-chain fatty acids was assessed in order to improve tolerance and productivity of these molecules for industrial applications. Nine proteins were identified as being differentially expressed under octanoic acid stress. Subsequent studies of deletion and overexpression mutants for the selected proteins led to identification of OmpF, an outer membrane porin, as

having the largest effect on fatty acid tolerance. OmpF was proposed as a transporter of fatty acids across the cell membrane, which can facilitate its import when present in the extracellular medium, therefore causing a decrease in intracellular pH. Utilization of glycerol by *E. coli* under anaerobic conditions was also studied in order to gain insights on the system level effect of this unconventional but advantageous feedstock with potential to be used in chemical and fuel production. In this study, wild-type *E. coli* was compared to the *adhE* deficient mutant under glycerol-fermenting and non glycerol-fermenting states. Twenty-six differentially expressed proteins were identified as being involved in known metabolic routes for glycerol utilization, while others suggested that amino acid metabolism, and phosphate and magnesium levels play key roles in glycerol fermentation. Lastly, the effects of mild hypothermia on the proteome of CHO cells were also studied with the goal of understanding metabolic state changes in response to a lower culture temperature. Eight differentially expressed proteins were identified suggesting that secretion and redox processes are the main systems affected by mild hypothermia. The studies presented in this thesis validated the potential of proteomics as a tool for characterizing metabolic systems and identifying targets for the engineering and development of viable biofactories.

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The past six years have been filled with great experiences that I will remember and value for years to come. I am grateful for everyone who has contributed scientifically and emotionally to the completion of this thesis, but I dedicate it to the support network who have pushed me to become better everyday,

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Chapter 1

Introduction

1.1. The era of biofactories

Thanks to the advances of the genomic revolution, a significant number of genomes have been sequenced and numerous genetic tools continue to be developed. Metabolic and cellular engineering have consequently become robust approaches for the development of biofactories, consisting of microbial or mammalian cell cultures engineered to produce molecules with desired traits for therapeutic or industrial applications. Furthermore, advances in systems biology have yielded powerful tools for the analysis of thousands of cellular components in a single study. These tools have proven useful in a number of studies for improvement of cellular behavior, and there is still significant potential for their application to tackle current industrial

challenges.

This work presents the potential of proteomic analysis for the characterization of three cellular systems with the ultimate goal of developing of more efficient platforms for chemicals, biofuels, and biopharmaceutical applications.

1.1.1. The transition to biorenewables

The current instability of oil supplies and the continuous fluctuation of oil prices (Association, 2009) (Figure 1.1) have sparked widespread interest in alternative and biorenewable sources for energy and carbon-based chemicals. In addition, an increase in the demand for fuels, and consequently, an increase in the petrochemical industry activity have triggered greenhouse gas emissions, thus causing undesirable damage to the environment (Figure 1.2). These factors, which revolve around economical, environmental, and geopolitical concerns, are key in fostering interest on the use of renewable resources for the production of fuels and carbon-based chemicals in biorefineries. Similar to petroleum refineries, biorefineries could produce multiple fuels and chemicals from a single feedstock, namely biomass. In the case of biorefineries, by-product wastes, such as glycerol, which is generated from other biofuel production processes, can also be taken advantage of and be converted into value-added fuels and chemicals.

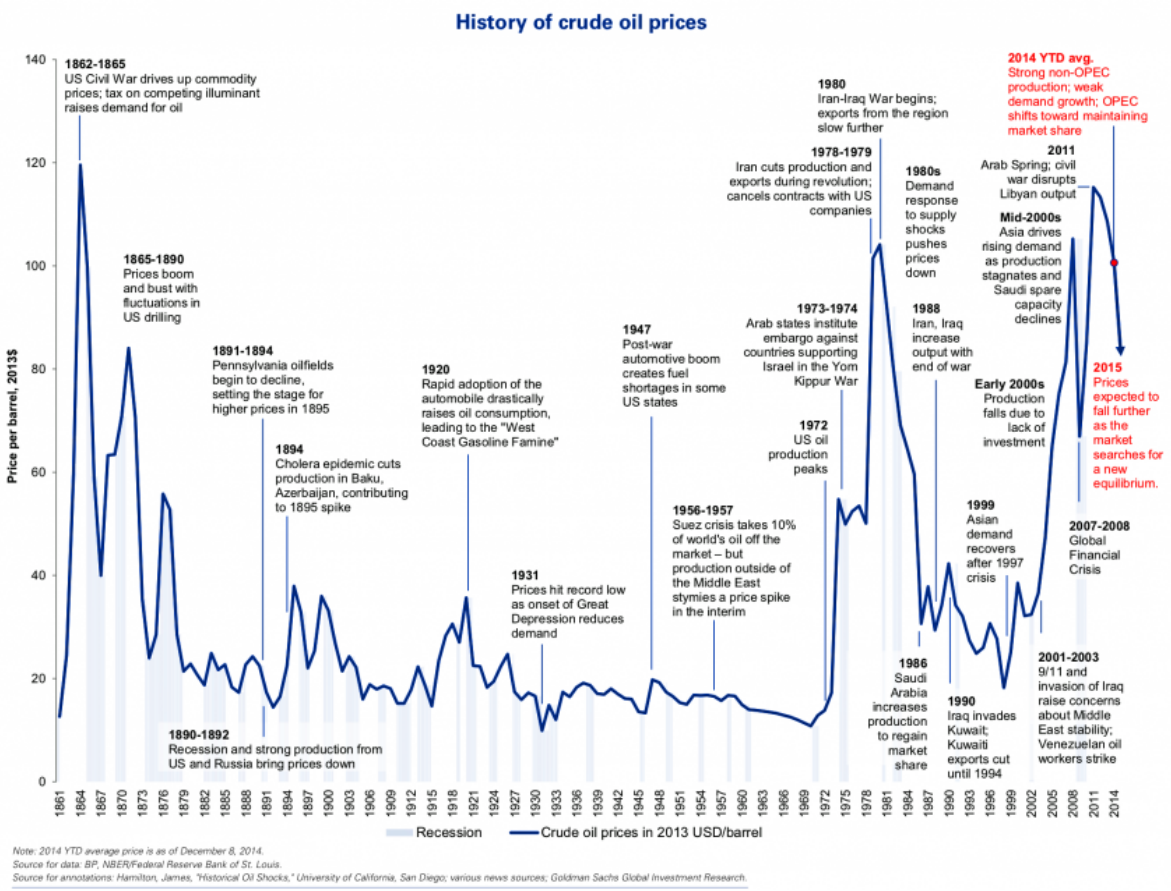


Figure 1.1. Historical trends of oil prices since 1861 (Ro, 2014).

In 2010, the Environmental Protection Agency (EPA) and the government of the United States announced the Renewable Fuels Standard, establishing a long-term renewable fuels production plan to be expanded from 9 billion gallons produced in 2009 to 36 billion gallons by the year 2022 (EPA, 2010). Growing world population and, consequently, increasing energy demands and greenhouse gas emissions present a great challenge and serve as motivation for the development of efficient platforms for the production of fuels and chemicals derived from biorenewable sources.

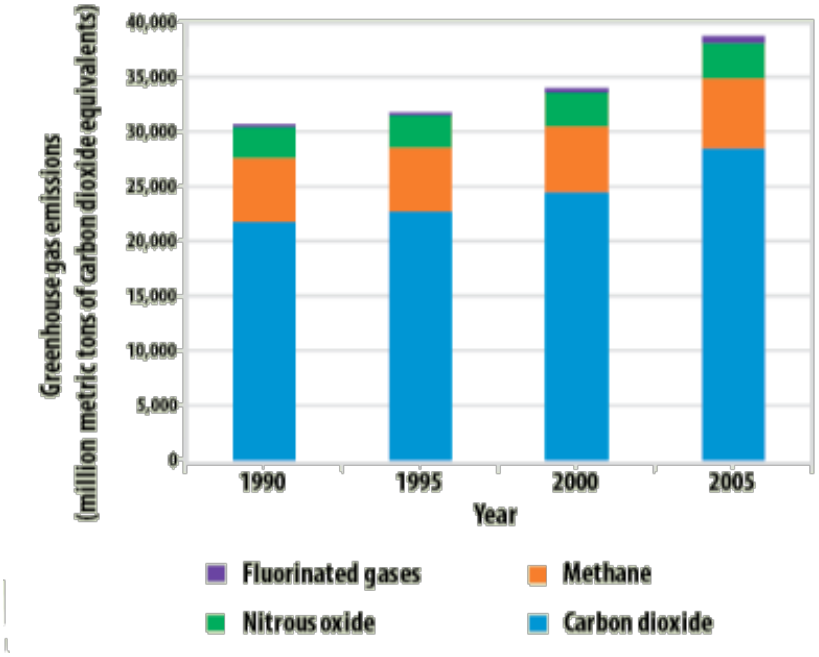


Figure 1.2. US Greenhouse gas emissions by gas, from 1990-2005. CO_2 emissions have increased from 1990-2005. An effort to decrease greenhouse gas emissions to reach 6,000 million metric tons by 2020 is being followed by public and private sectors (EPA, 2014).

In addition, while other energy-demanding processes (e.g. electricity generation) can be supplied by sources of alternative energy such as wind, solar, geo- and ocean- thermal, and hydroelectric (Rath, 2009), biomass represents the only renewable source for the production of carbon-based chemicals. Current processes for the conversion of biomass to chemicals primarily involve biochemical and thermochemical technologies (McKendry, 2002). The diversity of accessible biomass feedstocks, and the availability of different conversion technologies, make the bio-based product industry a viable substitute for petrochemical refineries. Corn and sugarcane are the

most common feedstocks currently used in the biotechnological industry and serve as potential feedstocks for a great range of biorenewable chemicals (Smith, 2008). Although markets for ethanol and biodiesel, currently the main biofuels, are increasing, the global potential for biorenewable fuels and chemicals is largely underused, particularly in some regions where there is significant potential for efficiency gains in both agricultural production and conversion to biofuels. Diversification from traditional carbon sources and fuel products is crucial for advancing the development of biorefineries. Investigating new carbon sources and chemical products could help achieve the desired environmentally friendly processes in a cost-efficient manner.

1.1.2. The quest for biopharmaceutical expansion

The market of biopharmaceuticals has been constantly increasing in the past decades, covering a large part of the pharmaceutical industry (Butler and Meneses-Acosta, 2012). But a rapid increase in the number and demand of approved biopharmaceuticals in recent years has in turn caused a shortage of production capacity (Thiel, 2004) (Figure 1.3). This shortage in production capacity has powered the design and construction of more efficient production facilities, as well as research strategies for the development of more productive mammalian cell systems. Among the areas that are currently being investigated with promising results are the development of vector

systems to allow high specific protein expression, downregulation of genes leading to apoptosis, characterization of glycosylation processes to improve consistency of glycoforms and enhance biological activity, and characterization of cellular metabolism and physiology.

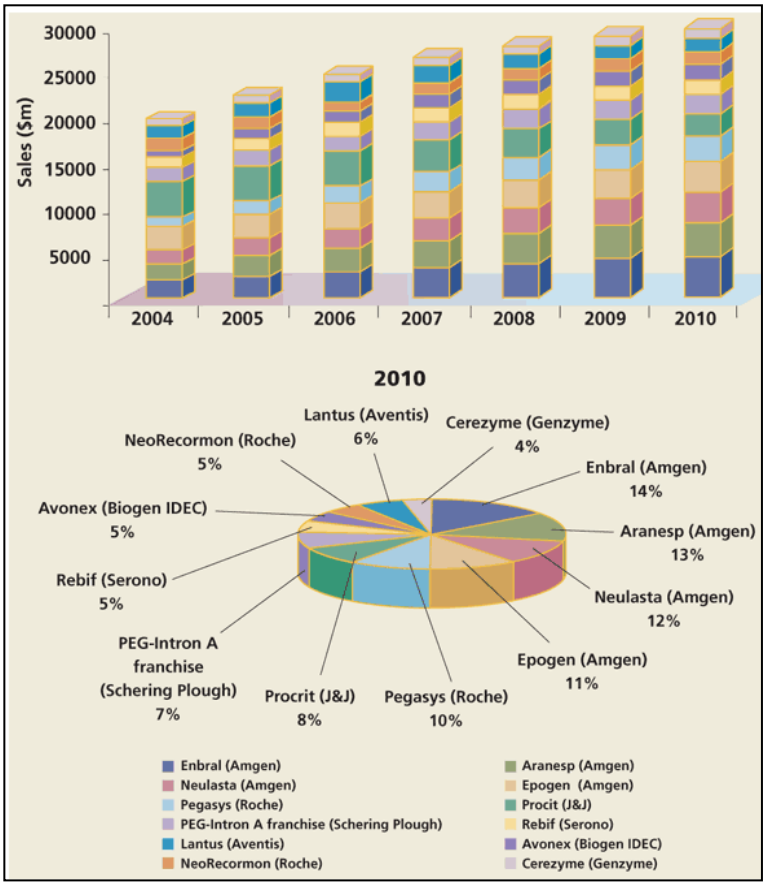


Figure 1.3. Market increase for leading recombinant proteins. An increase in the number and demand of biopharmaceutical products has caused a shortage of production capacity (Pavlou and Reichert, 2004).

The use of mammalian cell cultures for the production of biopharmaceuticals boomed in the 1950s with the design of large-scale

bioprocesses for human viral vaccines (Kretzmer, 2002) and they are still widely used in the biopharmaceutical industry for the production of a variety of biological molecules. However, recent interests in mammalian cell processes have focused on the production of recombinant proteins. Chinese hamster ovary (CHO) cells are the most widely used mammalian cell line for the production of recombinant proteins. Starting with the production of the recombinant human tissue plasminogen activator (rhtPA), CHO cells have been studied and manipulated for the production of other recombinant proteins (Butler and Meneses-Acosta, 2012). Developments in culture media, conditions, and equipment, as well as recent advances in metabolic engineering and systems biology will power further improvement of biopharmaceutical production processes for a more efficient and sustainable market.

1.2. Microbial and mammalian cell systems: the workforce of the post-genomic revolution

As a result of the post-genomic revolution of the last few decades, the use of microorganisms as cell factories has become an attractive alternative for the production of fuels and platform chemicals. The abundant knowledge on the genomes of industrial microorganisms like *Escherichia coli* and *Saccharomyces cerevisiae* and the recent sequencing of more than six hundred

genomes (527 bacterial, 47 archaeal, ad 65 eukaryotic) (http://genomesonline.org)(Liolios et al., 2008) have opened new opportunities for engineering cellular systems for the production of chemicals and biopharmaceuticals. The bacterium *E. coli* is among the most accessible microorganisms for the production of biorenewable chemicals, as it possesses very desirable traits. *E. coli* is amenable to large-scale fermentations and shows efficient growth at industrially relevant conditions. It is able to grow in mineral salts medium with inexpensive components and in the absence of oxygen, thus leading to lower fermentation and separation costs. *E. coli* can utilize a wide range of substrates from biomass origins, such as carbohydrates, polyols, and fatty acids. Its high growth and metabolic rates makes it suitable for attaining high productivity and yields. Also, the availability of genetic tools and the large amount of genomic, metabolic, and physiological knowledge has facilitated the engineering of this organism into efficient biocatalysts for the production of a broad range of chemicals (Baez-Viveros et al., 2004; Park et al., 2007; Shen and Liao, 2008). Similarly, Chinese hamster ovary (CHO) cell lines have become key for the manufacturing of therapeutic proteins (Jayapal et al., 2007). Their adaptability to suspension cultures in serum-free media satisfies safety and process requirements for the modern industry. In addition, their plasticity in terms of genetic modifications make them attractive for cellular engineering strategies.

Traditionally, industrial organisms have been developed by classical strain engineering techniques, which consist of multiple cycles of mutagenesis and selection in which the desired phenotypes are resolved (Sauer, 2001). However, new advances in metabolic engineering and synthetic biology have facilitated pathway engineering and strain optimization for the construction of new cell factories (Kern et al., 2007; Lee et al., 2008b). Moreover, new progress in the area of systems biology has given rise to new knowledge that assists in the development of cellular systems for industrial applications (Kuystermans et al., 2007; Mukhopadhyay et al., 2008). Systems biology provides information about genome-scale or cell-wide processes by combining high-throughput experimental tools in the areas of genomics, transcriptomics, proteomics, metabolomics, and fluxomics with global and predictive mathematical models of cellular metabolism (Lee et al., 2005).

1.3. Proteomic analysis to guide metabolic engineering strategies for cellular improvement

Improvement of yield, productivity, and titer, expansion of substrate and product range, and reduction of undesired by-products are a few of the goals that can be achieved by the application of metabolic engineering and systems biology tools to industrial organisms (Kern et al., 2007). Proteomics is

one of the technologies used in functional genomics to, not only identify and quantify differentially expressed proteins, but also guide studies to determine protein location, modifications, interactions, activity, and function. The first proteome study, reported by O'Farrell in 1975 (Ofarrell, 1975), introduced two-dimensional gel electrophoresis as a potential technique for protein separation. Presently, gel-based approaches for protein separation also include two-dimensional difference gel electrophoresis, in which proteins are tagged with fluorescent dyes prior to separation and, therefore, several samples can be run in one gel.

To date, two-dimensional gel electrophoresis is still one of the most used techniques for proteomic analysis, allowing separation of protein mixtures by two distinct properties: isoelectric point and molecular weight. Although originally, two-dimensional gel electrophoresis was used as a descriptive tool, the invention of soft ionization methods for biomolecules in mass spectrometry analysis, namely matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) has enabled the identification of proteins and detection of post-translational modifications. In these technologies, proteins or peptides are ionized and then analyzed by a time-of-flight (TOF) or ion trap mass analyzer to generate peptide mass fingerprints. Continued improvements in two-dimensional gel electrophoresis and mass spectrometry, and the development of powerful data analysis software have

enabled the use of proteomic analysis as high-throughput technologies. Figure 1.4 shows an overview of the experimental workflow for proteomic analysis in which (1) proteins are extracted, purified, and solubilized from control and experimental samples; (2) purified proteins are resolved by isoelectric focusing (IEF) and sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE); (3) protein expression is determined by image analysis using specialized softwares; (4) proteins of interest are extracted and identified by mass spectrometry.

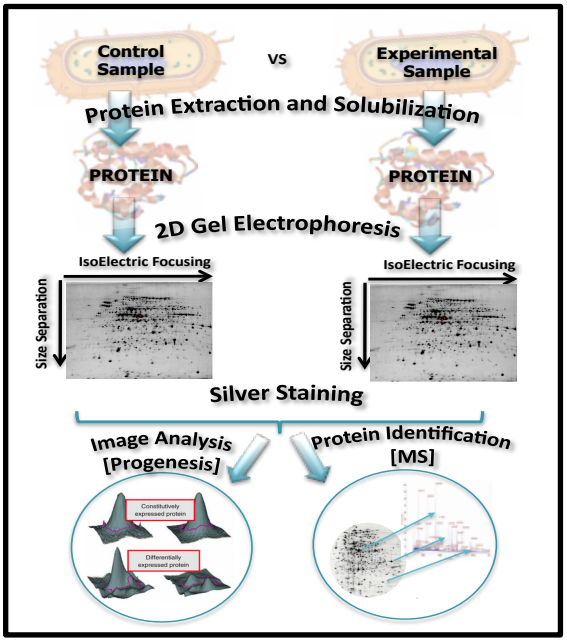


Figure 1.4. Overview of proteomic analysis.

This thesis presents the use of proteomic analysis to characterize different aspects of metabolism that could ultimately result in improved productivity and consumption rates. These studies are defined as three

separate chapters in this thesis and are briefly introduced in the following sections:

i. Proteomic analysis of the response of *Escherichia coli* to short-chain fatty acids
ii. Proteomic analysis of the fermentative metabolism of glycerol in *Escherichia coli*
iii. Proteomic analysis of Chinese hamster ovary (CHO) cells under mild hypothermia

1.3.1. Improving tolerance: Short-chain fatty acid toxicity in *Escherichia coli*

Given their simple and easy-to-manipulate chemical structures, short-chain fatty acids (SCFAs) are valuable feedstocks for many industrial applications. Short chain (C_6-C_{10}) fatty acids (SCFAs) have a wide range of applications, including artificial flavors (Jafari et al., 2008), pharmaceutical agents (Prajapati et al., 2012), and plasticizers for medical and commodity plastics (Mehta et al., 2014). They can be converted into α-olefins directed towards polymer synthesis (Espinosa-Gonzalez et al., 2014; Nikolau et al., 2008). Their relatively simple chemical structures allow better manipulation and control of carbon backbones in polymers, as well as easier incorporation of functional groups that confer desired properties to the molecule. Short-

chain FAs are also widely used as antimicrobal agents in the food, cosmetics, and pharmaceutical industries (Desbois, 2012). It is this antimicrobial quality that creates a limiting factor in the design of industrial strains with high SCFA productivity and yields (Jarboe et al., 2013; Lennen et al., 2011). While the microbial production of SCFAs by engineered *Escherichia coli* has been demonstrated recently, productivity and yields are still limited by these antimicrobial properties.

Limited production of the desired chemicals and reduced tolerance to toxic metabolites, including the desired products, are among the obstacles that can be encountered throughout the process of engineering microbial strains. Overcoming these obstacles begins by adequately characterizing and understanding the inhibition and production mechanisms of potential microorganisms for the production of the desired chemicals.

This work covers the first known proteomic analysis to assess the inhibitory effect of SCFAs in *E. coli*, via the following approaches:

i. Characterizing the response of *E. coli* to inhibitory concentrations of short-chain fatty acids

ii. Investigating differential protein expression in *E. coli* under short-chain fatty acid stress via two-dimensional gel electrophoresis

iii. Establishing the role of differentially expressed proteins on fatty acid toxicity mechanisms by testing genetic modifications (gene deletions and overexpression) and chemical assays

1.3.2. Diversifying feedstock availability: Fermentative utilization of glycerol in *Escherichia coli*

Glycerol has gained increased attention as a substrate for biorefineries in recent years, due to an increase in the production of biodiesel and bioethanol, both of which co-produce glycerol as a by-product. Its high degree of reduction also makes it a valuable carbon source for the production of reduced chemicals. However, highly reduced glycerol is difficult to metabolize by microorganisms without an external electron acceptor (i.e., fermentative conditions). In order to make this a viable process, substantial work has been done in the past decade to understand and characterize glycerol fermentative pathways in *E. coli*. Recent studies of glycerol fermentation in *E. coli* have achieved production of a variety of chemicals, including 1,2-propanediol, ethanol, succinate, and lactate, among others. New kinetic and genome-scale *in silico* models have also revealed key information about flux control during glycerol utilization by *E. coli.*

While substantial work has been done to understand, characterize and exploit the potential of glycerol as a feedstock for the microbial production of

a wide range of chemicals, they have followed a bottom-up approach, focusing on engineering specific enzymes and pathways.

In this chapter, the first system level comparison of the proteome of *E. coli* in different glycerol availability environments is intended to provide a better understanding of glycerol metabolism and identify targets for its improvement and expansion. This work was achieved by the following approaches:

i. Characterizing glycerol consumption in wild-type and mutant *E. coli* strains with fully functional and impaired glycerol utilization pathways

ii. Investigating differential protein expression in *E. coli* during glycerol fermentation via two-dimensional gel electrophoresis

iii. Developing informed hypothesis on the role of differentially expressed proteins on glycerol fermentation and potential ways to engineer *E. coli* for improved utilization of glycerol

1.3.3. Understanding cellular behavior: CHO cell metabolism under mild hypothermia

Recombinant protein therapeutics provide innovative and effective therapies for numerous illnesses, ranging from cancers to infertility. Mammalian cell cultures are widely used for the production of

recombinant proteins due to their ability to develop processes for correct assembly, folding and post-translational modifications. Despite the availability of a great number of cell lines, nearly 70% of all recombinant proteins are produced by CHO cells. The first recombinant therapeutic protein produced in CHO cells was rhtPA, which was approved for clinical use in 1987 (Jayapal et al., 2007). While there are plenty recombinant proteins being commercially produced, the abovementioned market challenges have sparked interest in developing more efficient cellular systems and facilities for improved production. Several studies in CHO cell batch cultures have identified mild hypothermia (culture temperatures between 30-33°C) as one of the variables responsible for increasing the production of recombinant proteins. This variable has been studied in batch cultures, but the inability to uncouple culture temperature and specific cell growth under this culture condition has made it difficult to assess specific gene or protein targets responsible for the increased productivity. Chemostat cultures allow the separation of these two variables by fixing the specific growth rate with a set dilution rate. Studying the effect of temperature as an individual variable on cell metabolism will elucidate which mechanisms are affected by this variable and will provide targets for engineering more robust CHO cell lines.

In this chapter, the first proteomic analysis of CHO cells in chemostat cultures under mild hypothermia is intended to provide better understating of cellular behavior at lower culture temperatures and to identify targets for future engineering efforts to increase the productivity of recombinant proteins. This work was achieved by:

i. Investigating differential protein expression in CHO cells grown under culture temperatures of 33°C and 37°C

ii. Developing informed hypothesis for the cellular systems affected by mild hypothermia

Chapter 2

Systems Biology approaches in Metabolic Engineering

2.1. Functional genomics and systems biology

Thanks to the advances of the genomic revolution, a significant number of microbial genomes have been sequenced (http://genomesonline.org) (Liolios et al., 2008), opening new opportunities for the challenging task of assigning biological function to each gene and elucidating the metabolic and regulatory networks that operate in a biological system. Of special relevance is the emergence of functional genomics, which concerns with "the development and application of high-throughput global (genome-wide or system-wide) experimental approaches to assess gene function by making use of the information and reagents provided by structural genomics" (Hieter and Boguski, 1997). The field of functional

genomics has grown exponentially in the last decades, embracing areas such as transcriptomics (mRNAs), proteomics (proteins), metabolomics (metabolites), and fluxomics (fluxes), among others (Figure 2.1).

Functional genomics represents the "experimental arm" of systems biology; the latter, being an approach to study biological systems by performing systematic (environmental and genetic) perturbations, monitoring global responses at molecular level (e.g. gene and protein expression, metabolite levels, and activity of metabolic and regulatory pathways), and integrating the obtained data for the formulation of predictive mathematical models that describe the system structure and its response to individual perturbations (Ideker et al., 2001a). As such, systems biology combines high-throughput experimental approaches that simultaneously analyze all the components of a biological system (collectively functional genomics approaches) (Ishii et al., 2007) with holistic models that predict the system behavior (Koide et al., 2009) (Figure 2.1). This integrative approach to understand and harness complex metabolic and regulatory networks is the basis to design more efficient cellular systems for the production of valuable molecules, such as biofuels, chemicals, and biopharmaceuticals.

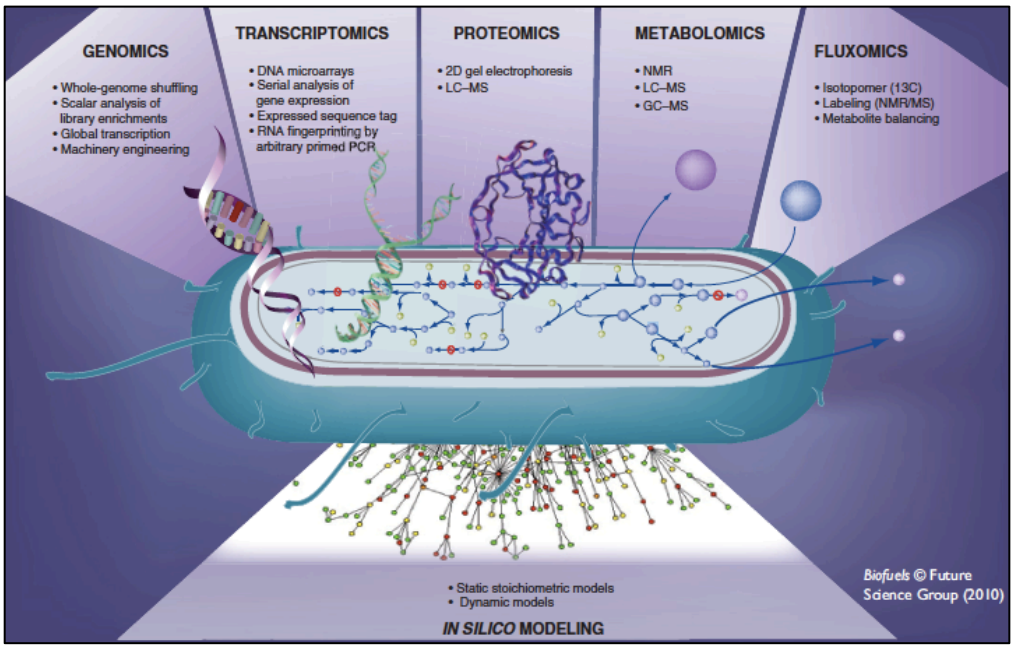


Figure 2.1. Representation of systems biology tools and analyses that power the harnessing of microbial metabolism. New knowledge about genome sequence space has stimulated advances in other SysBio areas. Technologies such as DNA microarrays have been used to characterize transcriptional responses of cellular systems to genetic and environmental perturbations. Similarly, proteomic profiling studies using GC or LC-based methods combined with mass spectrometry have allowed the identification and quantification of proteins and the assessment of variations in protein expression between different strains or under different environmental conditions. Finally, metabolomics and fluxomics tools have facilitated the quantification of intra- and extracellular metabolites and the distribution of metabolic fluxes inside the cell, respectively. Integration of these high-throughput tools has enabled the development of static and dynamic *in silico* models that describe and predict cellular behavior at a systems level(Rodriguez-Moya, 2010).

2.2. Metabolic engineering in the postgenomic era

While most industrial microbes have been traditionally developed by a process of mutagenesis and selection (Sahm et al., 1996; Tsuchida and Momose, 1986; Vertes et al., 2005), metabolic engineering provides modern tools for the development of well-defined cell factories (Chotani et al., 2000;

Vemuri and Aristidou, 2005). As defined by late James E. Bailey, metabolic engineering is "the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology" (Bailey, 1991). With help of metabolic engineering, the introduction of new pathways into host microbial organisms, and the optimization of existing ones, in order to achieve the production of fuel-grade chemicals have become accessible practices (Atsumi and Liao, 2008; Ladygina et al., 2006; Liu et al., 2009; Mukhopadhyay et al., 2008).

By application of metabolic engineering strategies, microbes can be developed into efficient cell factories that produce exogenous chemicals in quantities beyond what native organisms have been evolved to produce (Liao and Higashide, 2008). The introduction of new products, the reduction or elimination of undesired by-products, the extension of the substrate range, and the improvement of yield and productivity in the cell are all examples of achievable goals with the use of metabolic engineering (Kern et al., 2007). Another important goal is the improvement of general cellular properties that will yield fitter cells to withstand harsh environmental conditions, such as the presence of toxic products (Stephanopoulos, 2007). All of the abovementioned goals involve some kind of genetic modification, overexpression or knockout of target genes that can be made possible with the help of modern metabolic engineering tools.

Metabolic engineering is an iterative cycle that consists of the following steps (Figure 2.2): (1) *genotype engineering*, which involves strain construction via rational or evolutionary approaches by introducing gene modifications, knockouts or overexpression; (2) *phenotype resolution*, which refers to fermentation and evolution profiling to resolve desired phenotypes; (3) *systems analysis*, involving the use of functional genomics tools to characterize wild-type and engineered strains; and (4) *integration and design*, which entails the integration of results obtained from the systems analysis and use of conceptual and mathematical models of microbial metabolism to design targets for genetic engineering. As can be seen in Figure 2.2, the systems biology tools and approaches described in the previous section can be formally integrated into the metabolic engineering cycle. This, in turn, facilitates the discovery of promising genes and pathways for the production of novel industrial biorenewable chemicals, the introduction of the selected pathways into non-native organisms, and the evolution of these organisms into efficient cell factories (Stephanopoulos, 2007).

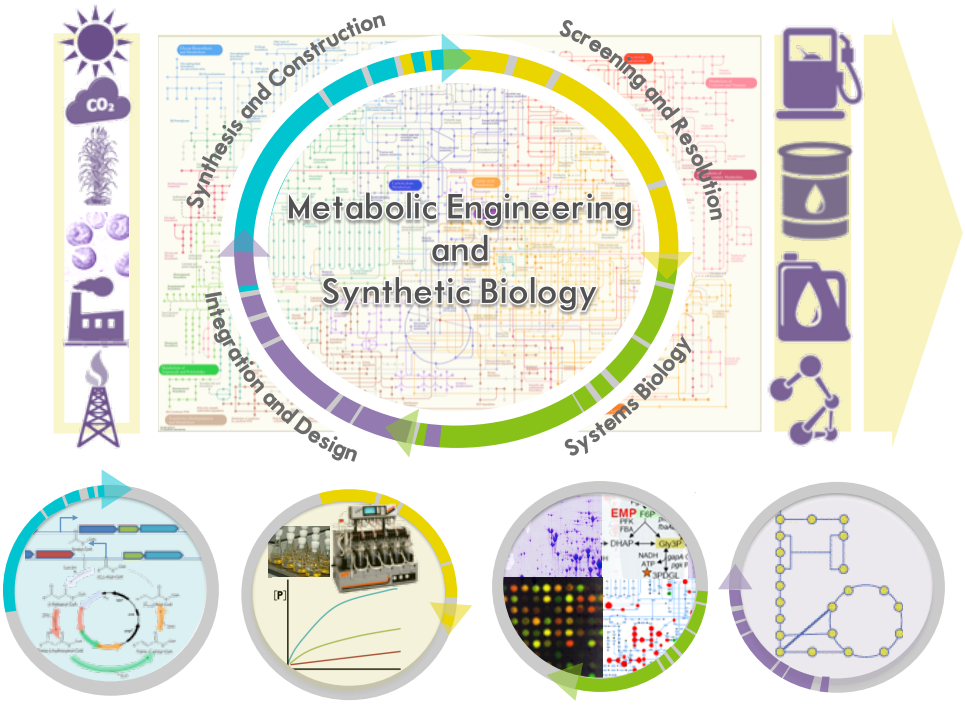


Figure 2.2. Metabolic engineering and synthetic biology cycle consisting of: (a) synthesis and construction, which involves pathway design and genetic modifications; (b) screening and resolution through fermentation and evolution profiling studies; (c) systems biology using functional genomics tools; and (d) integration and design through the use of conceptual and/or *in silico* models of microbial metabolism. Metabolic engineering is aided by systems biology tools in the genotype engineering, systems analysis and integration and design stages. Sources of carbon are shown on the left, while potential products are shown on the right.

Genetic engineering and molecular biology contribute to the metabolic engineering cycle by providing the tools to perform the necessary genetic modifications to the cell, while systems biology provides large amounts of genome-scale data, enabling the construction of mathematical models that describe and predict the impact of such genetic modifications on cellular behavior (Otero et al., 2007). The development of these models influence subsequent rounds of modifications to achieve the goals proposed

by metabolic engineering (Stephanopoulos and Stafford, 2002).

Another contribution of systems biology to strain development is in the context of inverse metabolic engineering, an approach used to optimize industrial microbes (Bengtsson et al., 2008; Jin et al., 2005; Stephanopoulos et al., 2005). In this approach, strains that overproduce a desired product are obtained via mutagenesis and selection, a technique also known as evolutionary engineering or metabolic evolution (Jantama et al., 2008). Functional genomics and systems biology tools are then used to identify mutations and metabolic changes responsible for the evolved phenotype. The data obtained from the functional genomics and systems biology analyses is then used for the rational implementation of genetic modifications, thus re-entering the metabolic engineering cycle described above.

Even though systems biology tools have been applied in the context of metabolic engineering for the production of native and foreign bioproducts, such as amino acids (Koffas and Stephanopoulos, 2005; Park and Lee, 2008), solvents (Senger and Papoutsakis, 2008a), vitamins (Shi et al., 2009b; van Ommen, 2004), and recombinant proteins (Lubke et al., 1995; Sommer et al., 2009), not until recently they have been used to study and optimize organisms involved in the production of biofuels and platform chemicals (Mukhopadhyay et al., 2008). Recent efforts for strain improvement aided by systems biology approaches are reviewed in detail in the next section and

summarized in Table 2.1.

Table 2.1. Application of systems biology for cellular improvement.

Systems Biology Technique	Organism	Description
Genomics	*Candida krusei*	Genome shuffling was used to improve acetic acid tolerance, and ethanol tolerance and production of the ethanologenic yeast(Wei et al., 2008)
	Escherichia coli	Genomic analysis using SCALEs for understanding genotype and identifying gene targets for improvement(Bonomo et al., 2008)
	Escherichia coli	Whole genome resequencing to monitor acquisition of mutations conveyed by growth adaptation to glycerol-based medium(Herring et al., 2006)
	Pichia stipitis	Whole genome resequencing for the identification of mutations and phenotypes for enhanced ethanol production from glucose(Smith et al., 2008)
	Saccharomyces cerevisiae	Genome shuffling to increase thermotolerance, ethanol tolerante, and ethanol productivity(Shi et al., 2009a)
	Saccharomyces cerevisiae	Global transcription machinery engineering for increased glucose and ethanol tolerance, and improved ethanol production(Alper et al., 2006)
Transcriptomics	*Bacillus subtilis*	Microarray analysis to study effects of genetic modifications, and culture conditions on gene expression (Kocabaş et al., 2009; Shilling et al., 2007)
	Clostridium acetobutylicum	Microarray analysis to study butanol stress and tolerance(Alsaker et al., 2004; Tomas et al., 2004)
	Clostridium acetobutylicum	Gene expression analysis to study effect of alcohol concentration on productivity(Tummala et al., 2003)
	Clostridium beijerinckii	Transcriptional analysis to identify target genes for butanol overproduction(Shi and Blaschek, 2008)
	Escherichia coli	Microarray study to investigate the mechanism of furfural inhibition in an ethanologenic strain(Miller et al., 2009a; Miller et al., 2009b)
	Escherichia coli	Microarray analysis to study ethanol, *n*-butanol, and isobutanol response networks(Brynildsen and Liao, 2009)
	Saccharomyces cerevisiae	Microarray analysis to study tolerance to high ethanol concentration(Dinh et al., 2009)
	Saccharomyces cerevisiae	Study of transcriptional regulation in central carbon metabolism(Daran-Lapujade et al., 2004)
	Chinese hamster ovary	Study of the effect of hyperosmotic stress on the productivity of an Fc-fusion protein (Shen et al., 2010)
Proteomics	*Clostridium acetobutylicum*	Proteomic study for identification of proteins involved in solvent production(Schaffer et al., 2002)
	Escherichia coli	Proteome analysis for the study of amino acid biosynthetic cycles that can lead to biofuel production(Kedar et al., 2007)
	Saccharomyces cerevisiae	Proteome profiling of chemostat cultures limited for glucose or ethanol(Kolkman et al., 2005)
	Saccharomyces cerevisiae	Comparative proteome analysis of xylose-utilizing strains(Karhumaa et al., 2009)

	Saccharomyces cerevisiae	Proteomic analysis of tolerance and adaptation to lignocellulosic inhibitory compound(Lin et al., 2009)
	Chinese hamster ovary	DIGE was performed to study the effects of sodium butyrate on and Bcl-x_L overexpression (Baik and Lee, 2010)
	Chinese hamster ovary	Proteomic analysis in serum-free medium supplemented with hydrolysates (Kim et al., 2011)
Metabolomics	*Escherichia coli*	Proteome analysis to study the effects of *fadR* gene knock-out leading to increased biomass yield(Peng and Shimizu, 2006)
	Saccharomyces cerevisiae	Comparative metabolome study under glucose-limited conditions(Mashego et al., 2005)
	Saccharomyces cerevisiae	Comparative metabolomic study between batch and continuous fermentation for improved ethanol production(Ding et al., 2009)
Fluxomics	*Escherichia coli*	Flux analysis of different perturbations on central carbon metabolism(Sauer et al., 1999)
	Saccharomyces cerevisiae	Comparative flux analysis of two xylose-fermenting recombinant strains(Grotkjaer et al., 2005)
	Saccharomyces cerevisiae	Metabolic flux analysis of xylose-fermenting strain exhibiting genes from *P. stipitis*(Pitkanen et al., 2003)
Intergrated Omics	*Escherichia coli*	Transcriptomic and proteomic analysis to obtain perturbation response data for construction of *in silico* model(Kashiwagi et al., 2009)
	Escherichia coli	Transcriptome and metabolome analysis for identification of targets for increased isoprenoid production(Kizer et al., 2008)
	Escherichia coli	Target genes for L-threonine over-production were identified by combined transcriptome profiling and *in silico* flux analysis(Lee et al., 2007)
	Chinese hamster ovary	Combined transcriptome and proteome analysis of low culture temperature for production of erythropoietin (Baik et al., 2006)
	Chinese hamster ovary	*In silico* model and metabolomic analysis to characterize fed-batch cultures (Selvarasu et al., 2012)
	Saccharomyces cerevisiae	Transcriptome and proteome studies of xylose-fermenting strain under aerobic culture conditions(Salusjarvi et al., 2008; Salusjarvi et al., 2006)
	Zymomonas mobilis	Transcriptome and metabolome profiling for increasing ethanol production(Yang et al., 2009)
***In silico* Models**	*Escherichia coli*	Elementary Network Reconstruction (ENR) of regulatory network behind carbon catabolite repression(Gonzalez, 2009)
	Escherichia coli	Model describes quantitative relation between primary carbon source uptake rate, oxygen uptake rate, and maximal cellular growth(Edwards et al., 2001)
	Escherichia coli	Improved model with more accurate description of *E. coli* K-12 metabolism(Reed et al., 2003)
	Saccharomyces cerevisiae	First comprehensive network of a eukaryotic organism(Forster et al., 2003)
	Saccharomyces cerevisiae	Metabolic model used to study pathway patterns for different combinations of glucose and oxygen uptake rates(Duarte et al., 2004)
	Zymomonas mobilis	Kinetic model that predicts ethanol production rate due to fermentation of different glucose and xylose mixtures(Altintas et al., 2006)

2.3. Application of systems biology tools for strain improvement

The following sections provide examples of the application of experimental and modeling approaches in systems biology to investigate cellular systems (Table 2.1).

Significant progress in the development of systems biology tools in recent years has powered the elucidation of complex phenotypes and the engineering of new ones for the development of promising industrial strains (Stephanopoulos et al., 2004; Zhang et al., 2006). Genomics tools enable the creation and enrichment of genomic libraries and help uncover the genotype-phenotype correlations that are proper of each organism, therefore contributing with new knowledge for strain improvement. Recently, genomics approaches have been used for the identification and analysis of the genetic basis of phenotypes from natural environments (Schmeisser et al., 2007) and from laboratory-engineered strains (Smith et al., 2008).

Transcriptomics and proteomics are powerful tools for the description of cellular physiology, and are the most commonly used for systems level analysis. Individual as well as combinatorial systems level studies using these techniques are presented below.

2.3.1. Transcriptomics

Transcriptomics, which is one of the main areas explored by systems

biology, focuses on studying the transcriptional response of organisms to genetic and environmental perturbations. Transcriptional profiling tools, such as DNA microarrays, enable comprehensive studies of gene expression, since the whole genome of an organism can be analyzed simultaneously while eliminating the unwanted biased selection of a subset of genes believed to regulate certain events (Dharmadi and Gonzalez, 2004). DNA microarrays study gene expression by identifying differentially expressed genes under different experimental conditions or resulting from certain genetic perturbations (e.g. comparison of wild-type and engineered strains). The results obtained from DNA microarray experiments can then be used to detect common expression patterns in groups of genes (Lockhart and Winzeler, 2000). The detection of differentially expressed genes can lead to the identification of potential genetic modifications in order to achieve production of the desired final product.

Gene array methods have been applied to *E. coli*, the workhorse for biotechnological applications, in order to obtain information about gene regulation in this organism under different substrate conditions. A study by Gonzalez *et al.* (Gonzalez et al., 2002) revealed differentially expressed genes between glucose- and xylose-fermenting cultures. The lower expression of genes involved in biosynthesis of small molecules, transcription, and translation in the xylose fermenting strains suggested why *E. coli* growth on

xylose was slower. Expression results also provided information about regulation of the Embden-Meyerhof-Parnas (EMP) and Pentose-Phosphate pathways (PPP) under the two growth conditions. Similar studies have been performed to compare gene expression in ethanol-tolerant and non-tolerant *E. coli* strains (Gonzalez et al., 2003) in order to determine the major genes responsible for increased tolerance to this biofuel. Tolerance appears to involve increased glycine metabolism and increased production of the osmolyte betaine, which suggest a connection between osmotolerance and ethanol tolerance. Other factors identified in the ethanol-tolerant strain were increased expression of genes for antibiotic resistance, serine uptake and deamination, loss of function of the global regulator FNR, and decreased production of organic acids.

Transcriptome analyses have also been performed in *Clostridium acetobutylicum* in order to study sporulation and physiology of this organism (Paredes et al., 2005). The mutant under study, which contains an inactivated butyrate kinase (*buk*) gene, starts butanol production significantly earlier and at higher levels than the wild-type strain (Harris et al., 2000). DNA microarray analysis of the strain and the M5 mutant, which is unable to sporulate or produce solvents due to the lack of the pSOL1 megaplasmid (Clark et al., 1989), revealed that up-regulation of solvent formation and stress genes were consistent with high levels of butyryl phosphate. These

studies also suggested that high levels of extracellular butyrate or acetate, or other types of stress, were not required for sporulation and solvent formation to start, as is the case for other solventogenic *Clostridia*.

Even though it is the most commonly sought goal in metabolic engineering, overproduction of a chemical that is not naturally produced, or not produced in large amounts, by an organism can cause toxicity to the cell. Organisms suffering toxic responses can be genetically modified in order to increase resistance to the perturbing substance. High levels of ethanol have been found to be harmful to *S. cerevisiae*, currently the main bioethanol producer in the biofuel industry, by affecting cell growth and productivity. Transcriptome profiling of two *S. cerevisiae* strains, one that showed active growth under ethanol stress and the other its parental strain, lead to the identification of genes that had an effect on ethanol resistance (Dinh et al., 2009), providing a better understanding of the tolerance of yeast to this common biofuel. Transcriptional analyses have also been performed in *C. acetobutylicum* to study the effect of gene *groESL* overexpression on butanol stress and tolerance (Tomas et al., 2004; Tomas et al., 2003).

Due to the high dimensionality of the data obtained by transcriptomic studies, it is usually difficult to visualize relationships between genetic and experimental conditions. Therefore, the application of data mining methods is crucial for efficient and accurate data analysis of gene expression data

(Bensmail and Haoudi, 2005). Different statistical and computational methods have been developed for reconstruction of transcription networks(Van den Bulcke et al., 2006; Wang et al., 2007), identification of gene clusters (Chen et al., 2005), and determination of assay-specific gene signatures (Rollins et al., 2006) from transcription data. These methods have proven to be effective for studying alcohol tolerance (Brynildsen and Liao, 2009; Rollins et al., 2006) and catabolite repression (Westergaard et al., 2007) in bacteria and yeast, both significant issues for the efficient microbial production of platform chemicals.

Gene expression and transcription data, coupled to gene knockouts and network component analysis (NCA), have been helpful in mapping the initial isobutanol response network of *E. coli* under aerobic conditions (Brynildsen and Liao, 2009). The resulting initial network was compared to those of ethanol and *n*-butanol in order to identify the common and distinct toxicity features of the three alcohols. The transcription factor ArcA was identified by NCA as the most significantly perturbed, and an *arcA* knockout confirmed it to be a major regulator of respiratory changes induced by isobutanol. NCA and the construction of similar response networks can assist in the design and comprehension of alcohol tolerance in the cell.

Principal Component Analysis (PCA) is another method that has been modified and applied to gene expression data mining. In one study, Rollins *et*

al. developed a method for identifying assay-specific gene signatures of an ethanol-resistant strain of *E. coli* (Rollins et al., 2006). Their approach combined the traditional PCA method with gene contribution plots to select the highest ranked genes of each assay, without incorrectly excluding genes by poor screening. In a different study by Westergaard *et al.*, PCA was combined with Analysis of Variance (ANOVA) and Student's t-test to pre-process gene expression data from glucose repression studies in *S. cerevisiae* (Westergaard et al., 2007). The transcriptional changes in the glucose transport and utilization pathways were mapped into the metabolic network by identifying the metabolites around which the most significant changes in transcription occurred (reporter metabolites). Although these methods showed to be effective for transcriptomic studies, they also present great possibilities for areas such as proteomics and metabolomics.

Even though transcriptomics offers powerful tools for the study of functional expression of the genomic information of microbial organisms, its scope is limited by not accounting for post-transcriptional processes (Dharmadi and Gonzalez, 2004). As a result, other omics tools such as proteomics, metabolomics, and fluxomics, should complement transcriptional studies in order to obtain the complete physiological description and production schemes of the desired cell factories.

2.3.2. Proteomics

In order to have an accurate description of the physiology and the regulatory networks in the cell, transcriptomic studies need to be complemented by other systems biology tools. Since most of the cellular activities are mediated by proteins, proteomics plays an important role in system level studies, and is the primary tool used to describe cellular interactions beyond mRNA level (Graham et al., 2007). Protein quantification and identification can be obtained by one- or two-dimensional gel electrophoresis, followed by software aided quantification tools and mass spectrometry techniques (Watt et al., 2003), or by gel-free techniques such as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and protein isotope labeling (Gevaert et al., 2007). Despite some disadvantages, two-dimensional (2D) gel electrophoresis is the most influential tool for the visualization of large sets of proteins and for the study of cellular behavior by comparative proteomic studies. Comparative proteomic analyses detect differentially expressed proteins by variations in the intensities of labeled protein spots in the gel, under different genetic or environmental conditions. Since proteins regulate most metabolic activities in the cell, proteome profiling studies provide valuable insights on protein function, expression and interactions that are essential for understanding the behavior of microorganisms from a systems level perspective.

C. acetobutylicum has been target of many studies in order to improve butanol production. The proteome profiles of the ATCC 824 wild-type strain with and without a control plasmid, and one showing overexpression of spo0A were compared by two-dimensional gel electrophoresis and mass spectrometry in order to identify differentially expressed proteins (Sullivan and Bennett, 2006). Out of 2,081 protein spots, 23 were identified and classified as heat shock response, acid and solvent formation, transcription and translation proteins. Overexpression of spo0A led to higher abundance of proteins involved in glycolysis, translation, heat shock stress response, and energy production in the organism. From the 23 proteins that were identified, 5 of them (GroEL, Hsp18, Tpi, Bcd, and ChW16/17) were found at different locations on the gel, suggesting they had undergone post-translational modifications. This was a surprising result, since such modifications were not thought to be so common in *C. acetobutylicum*. The study also revealed that proteins GroEL and GroES, which can increase solvent production and tolerance when over-expressed, were highly expressed during the stationary phase.

Ethanol production in *S. cerevisiae* has also benefited from proteomic studies (Cheng et al., 2008). The protein profile of this industrial yeast revealed that the principal enzymes involved in the production of ethanol were distributed throughout the major metabolic pathways of the cell (i.e.

glycolysis, gluconeogenesis, and pentose phosphate pathways). Other enzymes related to stress responses, such as glucose and nitrogen limitations, were also studied in order to establish the optimal fermentation conditions for ethanol production. The great number of differentially expressed proteins revealed that a significant amount of reactions in the major metabolic pathways of the cell need to be engineered in order to optimize ethanol production. Proteomic analyses like this one, combined with enzyme activity assays can be used to understand the dynamic behavior of the cell under different fermentation conditions, as well as pinpointing targets for bioprocess improvement of platform chemicals.

The proteomic profile of *S. cerevisiae* has been studied under different fermentation conditions in order to determine which parts of the metabolism are more significantly affected by changes in culture conditions. A study conducted on aerobic chemostat cultures resulted in the identification of 400 proteins with changing expression levels between cultures under glucose and ethanol limitations (Kolkman et al., 2005). The study revealed that only proteins involved in central carbon metabolism showed significant changes in expression level. By comparing their results with mRNA data from previous studies, central carbon metabolism processes could be classified as being regulated at the proteome or at the transcriptome level. Another study performed on *S. cerevisiae* provided information about the adaptation and

tolerance of yeast to vacuum fermentation (Cheng et al., 2009). Yeast cells have to deal with stress factors that might affect cell growth during fermentation, such as accumulation of inhibitory products, and variations in membrane fluidity. In addition, during vacuum fermentation, they have to overcome stresses due to lack of oxygen and negative pressure. Qualitative and quantitative analyses allowed identification of spots corresponding to 68 different proteins that were differentially expressed during different stages along the fermentation process. Analysis of the results indicated that upregulated proteins were more influenced by the negative pressures caused by vacuum, rather than the lack of oxygen. Both of these studies provided valuable insight on the metabolic pathways and culture conditions of one of the most studied microbes for biotechnological applications.

Besides acquiring information about the metabolic states of an organism, other proteomic studies focus on identifying targets that can be engineered in order to improve productivity of a bioprocess. For example, a significant increase in the production of amino acids phenylalanine, tyrosine, and tryptophan, which was recorded for a *pykF* mutant *E. coli* strain (Kedar et al., 2007), was found to be consistent with ten upregulated protein spots identified by MALDI-TOF MS as key enzymes in the regulation of the aromatic amino acid biosynthetic and degradation pathways. Since production of the potential biofuel 2-phenylethanol (Atsumi et al., 2008; Keasling and Chou,

2008) has been proposed through the phenylalanine biosynthetic pathway, knowledge about these enzymes provides the ground for further improvement of the productivity of this alcohol.

Although *Clostridium thermocellum* is a potential organism for the commercial production of ethanol from fibrous biomass, its industrial applications are limited due to the growth inhibitory effect of ethanol, even at low concentrations. Comparative proteome analysis between the wild-type and an ethanol-adapted strain showed that the most significant changes in protein expression belonged to membrane proteins, which were under-regulated in the ethanol-adapted strain (Williams et al., 2007). This study revealed targets for metabolic engineering that can result in the development of new industrial strains with improved ethanol production.

Proteomic studies have proven key for characterization of mammalian cell cultures as well. CHO cells are currently the most widely used cell line for the production of recombinant proteins. The effects of hyperosmotic stress (Baik and Lee, 2010; Kim et al., 2012) and medium composition (Kim et al., 2011) have been analyzed by proteomic analysis. Kim *et al* (Kim et al., 2012) found alterations in proteins related to cellular metabolism, cellular architecture, protein folding, mRNA processing, and protein secretion due to hyperosmotic stress from glycine betaine addition. Other studies investigating medium composition have found that molecular chaperones and

proteins linked to cell proliferation have been among the groups affected by changes in the medium (Baik and Lee, 2010; Kim et al., 2011). These systems will be further investigated in order to develop strategies to improve CHO cellular systems for increased productivity of recombinant proteins.

Integrating transcriptomics and proteomics, as well as combining them with other omics tools will yield more thorough analyses and could facilitate the development of hypothesis supported by more detailed data.

2.3.3. Integrated functional genomics analysis

Integrated omics studies facilitate the elucidation of the correlations that exist between genes, proteins, and metabolites during the metabolic functioning of an organism. Even though formal integration of omics tools has not been fully established, there have been several successful studies based on combined approaches for the improvement of industrial strains. Seeing that omics tools endow meaningful results when applied individually, studies that are supported by a combination of these tools will unquestionably propel transcendental results. Some studies integrating different omics techniques have been applied for more accurate phenotypic descriptions of microbes that can be potentially used as producers of platform chemicals, due to their flexibility of substrates, products, and the available knowledge about their genomic compositions.

Isoprenoid biosynthetic pathways have been suggested as a scheme for the production of fuel-grade short-chain alcohols, such as isopentanol and isopentenol, that have been identified as potential gasoline fuel additives, and as substitutes for diesel and jet fuel(Atsumi et al., 2008; Fortman et al., 2008; Lee et al., 2008b). Previous modifications of *E. coli* for high isoprenoid production led to an imbalance in carbon flux and accumulation of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA), which resulted to be cytotoxic to *E. coli*(Martin et al., 2003). Integrated transcriptomic and proteomic analyses were performed on *E. coli* in order to uncover the toxicity mechanism of HMG-CoA. The analyses showed a large concentation of fatty acid precursors, indicating that HMG-CoA inhibited fatty acid biosynthesis, also leading to membrane stress(Martin et al., 2003). Additional knowledge on the major metabolic pathways in *E. coli*, and identification of bottlenecks could result in more accurate and faster approaches for strain improvement.

In a study by Borden and Papoutsakis (Borden and Papoutsakis, 2007), genomic library enrichment and transcriptional profiling were combined to identify enriched genes that conferred growth advantage under butanol stress conditions. Further characterization of the identified genes by a combination of gene over-expressions and DNA microarray transcriptional profiling will help determine their role in solvent tolerance.

Alsaker, *et al.*(Alsaker and Papoutsakis, 2005) performed DNA microarray analysis on *C. acetobutylicum* strains at different fermentation stages. Gene expression profiles of the wild-type strain were compared to those of the non-solventogenic M5 strain, which lacks megaplasmid pSOL1. The majority of pSOL1 megaplasmid genes and solventogenic genes presented a continuous increased expression at the peak of the solventogenesis stage. Expression of primary metabolism and heat shock genes was higher in the M5 strain. Transcriptomic analysis was validated by comparison with a previous proteomic analysis (Schaffer et al., 2002), where solventogenic and heat shock stress proteins were also highly expressed during the solventogenesis stage.

Engineered strains of *Zymomonas mobilis* can produce near theoretical yields of ethanol with high specific productivity from pentose and hexose sugars. Transcriptome and metabolome profiles of the ethanologenic *Z. mobilis* ZM4 strain(Seo et al., 2005) under aerobic and anaerobic glucose fermentations revealed new findings about culture conditions for improved ethanol production(Yang et al., 2009). High Profile Liquid Chromatography (HPLC), Gas Chromatography (GC), and Gas Chromatography-Mass Spectroscopy (GC-MS) analyses in the absence of oxygen, showed that ZM4 had a higher glucose consumption rate, higher growth rate, and produced ethanol as the major end-product, while DNA microarray analysis showed

that the gene *pdc*, which encodes for a key enzyme in ethanol production (pyruvate decarboxylase), and several genes from the Entner-Doudoroff (ED) pathway (*glk, zwf, pgl, pgk,* and *eno*) were thirty-fold highly expressed under anaerobic conditions. Metabolome analyses also showed that the ethanol produced under aerobic conditions was only 1.7% of that produced under the absence of oxygen. Both studies confirmed that the absence of oxygen during fermentation promoted ethanol productivity(Yang et al., 2009).

S. cerevisiae has been engineered for the conversion of lignocellulosic sugars into ethanol(Matsushika et al., 2009). Transcriptome and proteome profiles of a xylose-fermenting *S. cerevisiae* strain were compared to those of glucose-grown cells in aerobic batch cultures in order to identify the main differences between the metabolic pathways of both sugars(Salusjarvi et al., 2008). During xylose metabolism, the observed genomic profile showed that the expression levels of several genes that were subject to glucose repression were intermediate between those in glucose repressed and derepressed cells. This suggests that some genes needed for xylose fermentation might not be expressed at sufficient levels, therefore resulting on slower fermentation of xylose than glucose. Proteome analysis revealed that phosphorilation of several glycolytic isoenzymes presented different patterns in xylose and glucose grown cells, thus suggesting that regulation of glycolysis also occurred at a post-translational level(Salusjarvi et al., 2008).

Integrated omics analyses have also been applied to study the effects of several genetic and environmental perturbations on the galactose utilization pathway in the yeast *S. cerevisiae*(Ideker et al., 2001b). First, a representation of the metabolic pathway was derived from the genes, proteins, and small metabolites known from previous studies of *S. cerevisiae*. The effects of 20 different genetic and environmental perturbations to the system were quantified using genomic and proteomic analyses. Integration of the obtained data with the proposed metabolic pathway and other known physical interactions resulted in new hypotheses about the pathway, and was therefore followed by subsequent rounds of perturbations to test the new hypotheses. The integrated analyses allowed identification of almost 1,000 mRNAs and 289 proteins relevant to the galactose utilization pathway. Out of the total proteins detected, 15 were identified as being post-transcriptionally regulated. Similar studies could suggest the drawbacks or advantages caused by the utilization of different substrates in *S. cerevisiae*, and could result in the construction of cost-efficient pathways that use abundant, lower-cost lignocellulosic sugars.

Although there are still some limitations, recent developments on CHO cell genetic information have sparked studies using omics tools. A recent integrative transcriptomic and proteomic study in CHO cells was focused on the effects of lower culture temperatures and butyrate addition (Kantardjieff

et al., 2010). These two variables have been shown to increase recombinant protein productivity in batch CHO cell cultures (Seth et al., 2007a). The main functional alterations observed in this study were related to energy metabolism, secretory machinery, redox balance, and cytoskeletal elements. These results open up areas for cellular engineering approaches to further improve productivity of recombinant proteins.

2.3.4. *In silico* Modeling

The large amounts of data generated by high-throughput systems biology tools can be integrated and organized into useful *in silico* models that can predict cellular behavior under different genetic and environmental conditions(Joyce and Palsson, 2006). Metabolic models can be divided into two main categories: static stoichiometric models, which describe the steady state properties of the system and excludes time-dependent variables; and dynamic models, which describe the time-dependent metabolic and regulatory behavior of the cell(Lee et al., 2005). Even though dynamic models could provide more accurate descriptions of regulatory networks, the lack of *in vivo* kinetic information has prevented the development of more comprehensive models that include metabolic and genetic regulation networks(Ishii et al., 2004).

The construction of *in silico* models, which have been developed to

predict substrate preference, consequences of gene deletions, optimal growth patterns, outcomes of adaptive evolution, and shifts in expression profiles(Price et al., 2003), has opened many opportunities for fine-tuning with the use of high-throughput omics data in order to make them stronger tools for strain design and improvement(Koide et al., 2009). The development of computational algorithms for the comprehensive analysis and design of metabolic networks has been enabled by the availability of "static" stoichiometric and dynamic models of cellular metabolism.

Flux Balance Analysis (FBA), which is based on constructing a metabolic model based on the assumption that the cell uses the available resources to maximize its growth, is one of the most popular approaches for computational modeling(Raman and Chandra, 2009). Fermentation characterization and metabolic flux analysis studies of engineered *C. acetobutylicum* strains using FBA revealed the effects of inactivation of gene *solR* in solvent production(Harris et al., 2001). Strain SolRH, which had the inactivated *solR* gene, showed a higher glucose utilization rate and produced higher butanol, ethanol, and acetone concentrations. Further engineering of the strain, by introducing a plasmid-encoded copy of the alcohol/aldehyde dehydrogenase (*aad*) gene used for butanol production, resulted in production of even higher concentrations of butanol, ethanol, and acetone than the SolRH strain. These results illustrate the potential for the design of

superior solvent-producing strains through targeted engineering of key regulatory genes.

Several attempts have been made to describe intracellular metabolism in *C. acetobutylicum* through the construction of *in silico* models. The model developed by Lee, *et al.*(Lee et al., 2008a), which included a combination of 502 reactions and 479 metabolites, used FBA and non-linear programming to describe the acidogenic and solventogenic phases, respectively. This model was developed to examine the potential for enhanced butanol production. Gene knockout simulations can also provide qualitative and quantitative predictions of the production rates of certain metabolites of interest(Senger and Papoutsakis, 2008a). Such predictions have proven to be useful in strain improvement for metabolite production. In another metabolic model developed for *C. acetobutylicum*, gene knockout simulations were studied to reveal essential enzymes for cellular growth (Senger and Papoutsakis, 2008a). The model was combined with thermodynamic analysis to determine the physiological conditions of the cell for the resulting metabolic states. The constraints set on mathematical models usually play an important role on the results given by the simulations. A new constraint was described and applied to a metabolic model of *C. acetobutylicum* in order to limit the amount of possible phenotypic solutions(Senger and Papoutsakis, 2008b). This constraint, named *specific proton flux state*, was imposed for the influx and

efflux of free protons to allow accurate predictions of the extracellular medium pH during vegetative growth of batch cultures.

One innovative approach for design and prediction purposes in *E. coli* was the development of a mathematical model that suggests gene deletion strategies that lead to overproduction of a desired chemical(Burgard et al., 2003). By coupling biomass formation with chemical overproduction due to stoichiometry, the optimization of one function directly relates to the other. This model was tested for overproduction of three different chemicals; lactate, succinate, and 1,3-propanediol. While some of the suggested strategies were intuitive, others suggested more complex mechanisms that could not have been easily perceived. Even though this is a powerful model for strain design and improvement, the lack of regulatory and kinetic information limits its applications.

A dynamic flux balance model was constructed to predict cell growth and ethanol production by *S. cerevisiae*, using glucose or xylose as substrate, and by changing from aerobic to anaerobic culture conditions(Hjersted et al., 2007). The model consisted of coupling kinetic uptake expressions and dynamic extracellular mass balances on substrates and metabolic products to intracellular flux balances. Analysis of several genetic modifications led to conclude that ethanol production was improved when microaerobic growth was allowed after switching from the aerobic phase. This model helped

analyze existing and discover new engineering strategies for improvement of ethanol production from glucose and xylose(Hjersted et al., 2007).

Kinetic and genome-scale models for glycerol metabolism in *E. coli* have been developed in our group (Cintolesi et al., 2014; Cintolesi et al., 2012) in order to obtain additional information about glycerol metabolism that may be more difficult and time-consuming if done experimentally. The kinetic *in silico* model, combined with metabolic control analysis (MCA) revealed key information about the enzymes controlling the flux of carbon during glycerol fermentation by *E. coli.* Enzymes DhaK and GldA were identified by this analysis and experimental overexpression of these enzymes resulted in an increase in ethanol production (Cintolesi et al., 2012). In addition, a genome-scale model simulating the reversal of the β-oxidation cycle provided valuable information about the production of a great range of fatty acid-derived molecules (Cintolesi et al., 2014).

Databases, such as CellML (http://www.cellml.org)(Lloyd et al., 2008), E-Cell (http://www.e-cell.org) (Tomita et al., 1999) and SBML (http://www.sbw-sbml.org)(Stromback and Lambrix, 2005), have started to collect dynamic data and models that can enable further development of more complex kinetic models(Snoep and Olivier, 2003). One example was the development of a mathematical model describing glycolysis in *S. cerevisiae*(Rizzi et al., 1997). Incorporation of the rate equations for the

reactions into the model led to predictions of changes in intra- and extra-cellular metabolite concentrations corresponding to a glucose pulse. This model, which predicts stationary, as well as time-dependent metabolic states, can provide crucial information about the behavior of *S. cerevisiae* under different conditions, therefore reducing the need for time-consuming experiments that lead to the same conclusions(Rizzi et al., 1997). Even though limitations of kinetic data have delayed the development of dynamic models, recent advances in bioinformatics, computational biology and systems biology are facilitating new tools that will lead to an increase of kinetic models describing complex biological systems (Ishii et al., 2004).

Chapter 3

Materials and Methods

Chapter 3 presents general materials and methods that were used throughout the thesis. Descriptions of culture medium and conditions specific to each of the subsequent chapters, as well as particular modifications to the methods, are discussed in each of the chapters.

3.1. *Escherichia coli* Strains, Plasmids, and Genetic Methods

All *Escherichia coli* strains, plasmids, and primers used in this study are listed in Supplementary Tables 1 and 2. Wild-type K12 *Escherichia coli* strain MG1655 (Kang et al., 2004) was used for the toxicity and proteomic analyses in the FA study. Wild-type K12 *E. coli* strain BW25113 and its derivatives from the Keio collection (Baba et al., 2006) were used to study glycerol fermentation and as hosts for all genetic modifications. Double gene

knockouts were introduced in BW25113 and its derivatives by P1 phage transduction. Single gene knockout mutants from the National BioResource Project (NIG, Japan) (Baba et al., 2006) were used as donors of specific mutations. All mutations were confirmed by polymerase chain reaction.

Gene overexpression was achieved by cloning the desired gene(s) in medium-copy based vectors (pTrcHis2A, abbreviated pTH_A; Invitrogen, Carlsbad, CA, or pTL_K) utilizing In-Fusion PCR cloning technology (Clontech Laboratories, Inc., Mountain View, CA). High-copy overexpression was obtained using the corresponding vectors (pCA24N, -*gfp*) from the ASKA collection from the National BioResource Project (NIG, Japan) (Kitagawa et al., 2005). Cloning inserts were created via PCR of ORFs of interest from *E. coli* genomic DNA using the primers listed in Supplementary Table S2 with Phusion DNA polymerase under standard conditions described by the supplier (Thermo Scientific, Waltham, MA). Vector backbone was purified from *E. coli* cultures (Qiagen, Valencia, CA) and digested with the restriction enzymes listed in Supplementary Table S2 as according to the manufacturer (New England Biolabs, Ipswich, MA) to enable cloning. The resulting In-Fusion products were used to transform *E. coli* Stellar cells (Clontech Laboratories, Inc., Mountain View, CA), and positive clones were confirmed by PCR, restriction digestion, and DNA sequencing. For pTL_K construction, the kanamycin ORF from pKD4(Datsenko and Wanner, 2000) was seamlessly

recombineered into vector pTL(Blankschien et al., 2010) to replace the ampicillin marker. Site-directed mutagenesis of the whole plasmid was then performed to eliminate the extra NcoI site introduced with the kanamycin marker(Sambrook and Russell, 2001).

All molecular biology techniques were performed with standard methods (Miller, 1972; Sambrook and Russell, 2001) or by manufacturer protocol. Strains were kept in 32.5% glycerol stocks at −80 °C. Plates were prepared using LB medium containing 1.5% agar, and appropriate antibiotics were included at the following concentrations: ampicillin (100 μg/mL), kanamycin (50 μg/mL), and chloramphenicol (34 μg/mL).

3.2. Analytical Methods

Optical density was measured at 600nm in a Thermo Spectronic Genesys 20 (Thermo Scientific, Waltham, MA). For all closed Hungate tube experiments (see Chapter 5), optical density was measured at 600 nm using a WPA BioWave CO8000 Cell Density Meter (Biochrom Ltd, Cambridge, England). Quantification of glucose, glycerol, and metabolic products in the culture supernatant was conducted via ion-exclusion high-performance liquid chromatography (HPLC) using a Shimadzu Prominence SIL 20 system (Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with an HPX-87H organic acid column (Bio-Rad, Hercules, CA) with operating conditions to

optimize peak separation (0.3 mL/min flow rate, 30 mM H_2SO_4 mobile phase, column temperature 42 °C).

When studying glycerol fermentation, one-dimensional (1D) proton nuclear magnetic resonance (NMR) analysis was used to confirm that no glycerol traces were found in the casamino acids used to supplement the medium. NMR spectrum of the casamino acid was compared to a 10g/L glycerol standard, with the following protocol: 60 µL of D_2O and 1 µL of 600 mM NMR internal standard TSP [3-(trimethylsilyl)propionic acid-D4, sodium salt] were added to 540 µL of the sample. The resulting solution was then transferred to a 5 mm-NMR tube, and 1D proton NMR spectroscopy was performed at 25°C in a Varian 500-MHz Inova spectrometer equipped with a Penta probe (Varian, Inc., Palo Alto, CA) using the following parameters: 8,000Hz sweep width, 2.8s acquisition time, 256 acquisitions, 6.3-µs pulse width, 1.2-s pulse repetition delay, and presaturation for 2s. The resulting spectrum was analyzed using FELIX 2001 software (Accelrys Software Inc., Burlington, MA). Peaks were identified by their chemical shifts and Jcoupling values.

3.3. Proteomic Analysis

3.3.1. Protein Extraction, Precipitation and Quantification

Cells were harvested and washed 3 times with a buffer containing 3mM KCl, 68mM NaCl, 1.5mM KH_2PO_4, and 9mM NaH_2PO_4 and centrifugation at 10,000rpm and 4ºC for 15 minutes. After washing, cell pellets were resuspended in 1mL of lysis solution (30mM Tris, 2M Thiourea, 7M Urea, 4% (w/v) CHAPS, pH adjusted to 8.5 with HCl) and left on ice for 10 minutes. Cell suspensions were sonicated at 10% power for a total of 10 minutes in a Branson Sonifier 250 (Branson Ultrasonics Co., Danbury, CT). Sonicated samples were centrifuged at 12,000rpm and 4ºC for 10 minutes. The supernatant containing proteins was then precipitated with the GE Healthcare 2D Clean-Up Kit (GE Healthcare and Bio-Sciences, Pittsburg, PA). The precipitated proteins were solubilized in a rehydration buffer containing 2M Thiourea, 7M Urea, 2% (w/v) CHAPS, 1.0% IPG Buffer pH 4-7, and a trace amount of bromophenol blue. Protein concentration was measured with the GE Healthcare 2D Quant Kit (GE Healthcare and Bio-Sciences, Pittsburg, PA), following manufacturer instructions.

3.3.2. 2-Dimension Electrophoresis Separation

Samples were diluted in rehydration buffer, such that 450µL of the final solution contained 100µg of proteins, for gels to be silver-strained, or 500µg, for gels to be stained with coomassie blue. 15mg/mL DeStreak reagent (GE Healthcare and Bio-Sciences, Pittsburg, PA) and 0.2% (w/v) DTT were added to the rehydration solution containing the proteins and the solution was incubated with an Immobiline DryStrip Gel (pH 4-7, 24cm, GE Healthcare and Bio-Sciences, Pittsburg, PA) at room temperature for 12-16 hours. Focusing was performed in an Ettan IPGphor system (GE Healthcare and Bio-Sciences, Pittsburg, PA) in 4 steps: (1) 500V for 1h (linear, 0.5kVh), (2) 1000V for 1h (gradient, 0.8kVh), (3) 10000V for 3h (gradient, 13.5kVh), (4) 10000V for 3h (linear, 30kVh). After focusing, the strips were equilibrated for 15 min by first incubating them in an equilibration solution (50mM Tris-HCl, 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and a trace of bromophenol blue, with pH adjusted to 8.8) with 1% (w/v DTT), followed by 15 min incubation in the equilibration solution with 2.5% (w/v) iodoacetamide. The equilibrated strips were transferred to DALT Gels 12.5 (26x20cm, GE Healthcare and Bio-Sciences, Pittsburg, PA), and separated in the second dimension in an EttanDALT*six* system (GE Healthcare and Bio-Sciences, Pittsburg, PA) at 25°C in 2 steps: (1) 2W/gel for 45minutes, and (2) 17W/gel for 3-4h. The running buffer for the bottom chamber contained 2.5mM Tris,

19.2mM Glycine, and 0.02% (w/v) SDS. The buffer for the upper chamber was 2X of the bottom chamber buffer.

3.3.3. Gel Staining

Gels used for comparison and statistical analysis were stained with PlusONE Silver Staining Kit for Proteins (GE Healthcare Bio-Sciences, Pittsburg, PA), following manufacturer instructions. Gels used for extraction of spots for MS-identification were stained with PlusONE Coomassie Tablets, PhastGel Blue R-350 (GE Healthcare Bio-Sciences, Pittsburg, PA), following manufacturer instructions.

3.3.4. Data Analysis

The stained gels were scanned, compared and analyzed using Progenesis Same Spots software (version 2.0.2733.19819, Nonlinear Dynamics, Newcastle, UK). The logarithms of the normalized spot volumes ($\log NV_n = \log [(V_n / V_t) \times 100]$, where NV_n is the normalized spot volume of spot *n*, V_n is the volume of spot *n*, and V_t is the total volume of all spots in the gel) were compared to determine the protein spots with a p-value ≤ 0.05 (ANOVA test). When more than one variable was being compared (see Chapter 5), the normalized spot volumes were analyzed via two-way ANOVA

in Matlab v7.11.0 (R2010b, The Mathworks, Inc.). Selected spots were excised from the gel and identified by MALDI-TOF/MS.

3.3.5. SDS-PAGE of Membrane Proteins

Harvested cells were washed with 0.9g/L NaCl and resuspended in 5mL of sodium phosphate buffer (0.125M Na_2HPO_4 and 0.125M NaH_2PO_4) with 0.1g/L lysozyme. Samples were incubated at 30ºC for 10 minutes and then sonicated at 5% power for 10 minutes in a Branson Sonifier 250 (Branson Ultrasonics Co., Danbury, CT). Sonicated samples were then centrifuged at 5000g and 4ºC for 10 minutes. The supernatants were transferred to ultracentrifuge tubes and centrifuged at 30000rpm and 4ºC for 1h in a Beckman-Coulter Optima L-80XP Ultracentrifuge with a Type 45Ti fixed rotor (Beckman-Coulter, Pasadena, CA). Protein pellets were resuspended in 0.5mL of sodium phosphate buffer.

Samples were analyzed using NuPAGE® Novex 12% Bis-Tris 10-well gels (Invitrogen, Carlsbad, CA), as described by the manufacturer. Once electrophoresis was completed, the gel was washed and stained with SimplyBlue™ SafeStain (Invitrogen, Carlsbad, CA), according to the manufacturer protocol.

3.4. Identification of protein spots by Mass Spectrometry Analysis

3.4.1. Sample Preparation

Gel samples were cut into 1mm size pieces or smaller and placed into separate 0.5mL polypropylene tubes. 100mL of 50mM ammonium bicarbonate buffer was added to each tube and the samples were then incubated at 37°C for 30min. After incubation, the buffer was removed and 100mL of water was added to each tube. The samples were then incubated again at 37°C for 30min. After incubation, the water was removed and 100mL of acetonitrile was added to each tube to dehydrate the gel pieces. The samples were vortexed, and after 5min the acetonitrile was removed. 100mL of acetonitrile was again added to each of the sample tubes, vortexed, and acetonitrile was removed after 5 minutes. The samples were then placed in a speedvac for 45 minutes to remove any excess solvent.

A 25mM ammonium bicarbonate solution was prepared at pH 8.0. A volume of 2mL of 25mM ammonium bicarbonate was added to a 20mg vial of lyophilized trypsin (Promega Corp.). The trypsin solution was then vortexed. Trypsin solution was added to each sample tube in an amount (approximately 10mL) to just cover the dried gel. The samples were then incubated at 37°C for 6h.

After digestion, the samples were removed from the oven and 1 mL of sample solution was spotted directly onto a MALDI target plate and allowed

to dry. 1 mL of alpha-cyano-4-hydroxycinnamic acid (Aldrich Chemical Co.) matrix solution (50:50 acetonitrile/water at 5 mg/mL) was then applied on the sample spot and allowed to dry. The dried MALDI spot was blown with compressed air (Decon Laboratories, Inc.) before inserting into the mass spectrometer.

3.4.2. MALDI-TOF/MS

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF/MS) was used to analyze the samples and determine protein identification. Data were acquired with an Applied Biosystems 5800 MALDI TOF/TOF Proteomics Analyzer. Applied Biosystems software package included 4000 Series Explorer v. 4.1.0 (build 12) with Oracle Database Schema Version (v. 4.0.0), Data Version (4.0.5) to acquire both MS and MS/MS spectral data. The instrument was operated in positive ion reflectron mode, mass range was 850-3000Da, and the focus mass was set at 1700Da. For MS data, 2000-4000 laser shots were acquired and averaged from each sample spot. Automatic external calibration was performed using a peptide mixture with reference masses 904.468, 1296.685, 1570.677, and 2465.199.

Following MALDI MS analysis, MALDI MS/MS was performed on 10 abundant ions from each sample spot. A 1kV positive ion MS/MS method was

used to acquire data under post-source decay (PSD) conditions. The instrument precursor selection window was +/- 3 Da. For MS/MS data, 2000 laser shots were acquired and averaged from each sample spot. Automatic external calibration was performed using reference fragment masses 175.120, 480.257, 684.347, 1056.475, and 1441.635 (from precursor mass 1570.700).

Applied Biosystems ProteinPilot™ software 4.0.8085 was used in conjunction with MASCOT to search the respective protein database using both MS and MS/MS spectral data for protein identification. Protein match probabilities were determined using expectation values and/or MASCOT protein scores. MS peak filtering included the following parameters: mass range 800Da to 4000Da, minimum S/N filter = 10, mass exclusion list tolerance = 0.5Da, and mass exclusion list (for some trypsin and keratin-containing compounds) included masses 842.51, 870.45, 1045.56, 1179.60, 1277.71, 1475.79, and 2211.1. For MS/MS peak filtering, the minimum S/N filter = 10.

For protein identification, *E. coli* taxonomy was searched in the National Center for Biotechnology Information (NCBI) database. Other parameters included the following: selecting the enzyme as trypsin; maximum missed cleavages = 1; fixed modifications included carbamidomethyl (C) for 2-D gel analyses only; variable modifications included oxidation (M); precursor tolerance was set at 0.5 Da; MS/MS

fragment tolerance was set at 0.5 Da; mass = monoisotopic; and peptide charges were only considered as +1. The significance of a protein match, based on both the peptide mass fingerprint (PMF) in the first MS and the MS/MS data from several precursor ions, was based on a p-value<0.05.

Chapter 4

Proteomic Analysis of the Response of *Escherichia coli* to Short-Chain Fatty Acids

4.1. Introduction: Short-chain fatty acids as building blocks for the chemical industry

Given their simple and easy-to-manipulate chemical structures, short-chain fatty acids (SCFAs) are valuable feedstocks for many industrial applications. Their relatively simple chemical structures allow better manipulation and control of carbon backbones in polymers, as well as easier incorporation of functional groups that confer desired properties to the molecule. Short-chain FAs are also widely used as antimicrobal agents, and it is this antimicrobial quality that creates a limiting factor in the design of industrial strains with high SCFA productivity and yields (Jarboe et al., 2013; Lennen et al., 2011). While the microbial production of SCFAs by engineered

Escherichia coli has been demonstrated recently, productivity and yields are still limited by these antimicrobial properties.

The following sections discuss the biosynthetic pathways to fatty acids in *Escherichia coli* and recent efforts in metabolic engineering to diversify the variety of products derived from fatty acid metabolic routes.

4.1.1. Fatty acid synthesis in *Escherichia coli*

Fatty acid synthesis in *E. coli* is carried out in three main stages: initiation reaction, elongation cycle, and termination (Figure 4.1). All the reactions involved in the synthesis of fatty acids are catalyzed by fatty acid synthase (FAS), a multienzyme complex. The different enzymes in the FAS complex act together to synthesize fatty acids from acetyl-CoA and malonyl-CoA through a series of reductive reactions in which NADPH acts as electron donor.

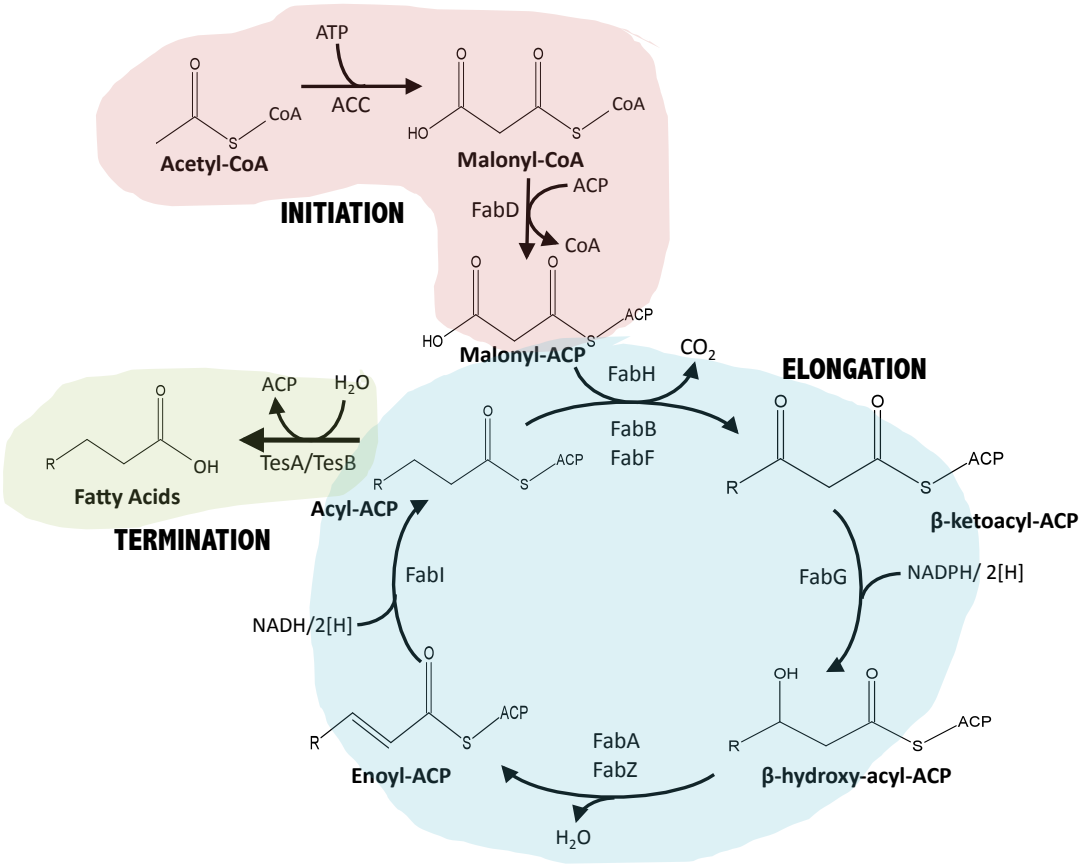


Figure 4.1. Fatty acid biosynthetic cycle. Enzymes catalyzing each reaction are shown. Abbreviation: ACP, acyl-carrier protein; R, carbon chain of different lengths, depending on the number of elongation cycles.

In the initiation reaction, malonyl-CoA, the first precursor for fatty acid synthesis, is formed from the acetyl-CoA pool in what has been identified as the first committed step of fatty acid synthesis (Cronan and Thomas, 2009). This reaction is the rate-limiting step in fatty acid synthesis since the levels of malonyl-CoA are directly proportional to the rate of production of fatty acids. Malonyl-CoA is then converted to malonyl-ACP by FabD. ACP is small and one of the most abundant proteins in *E. coli*, to which all intermediates during fatty acid synthesis are bound. The malonyl-ACP complex, together with an

acetyl-CoA molecule, is then ready to enter the elongation cycle for the synthesis of fatty acids.

The fatty acid elongation cycle starts with a condensation reaction in which a malonyl-ACP molecule reacts with an acyl thioester to form 3-ketoacyl-ACP, which is then reduced to 3-hydroxyacyl-ACP. The subsequent dehydration of 3-hydroxyacyl-ACP results in the production of an enoyl-ACP. Enoyl-ACP is then reduced to butyryl-ACP. This constitutes the end of the first cycle of fatty acid synthesis. At the end of each cycle, the saturated acyl-ACP molecule that is produced reacts with another malonyl-ACP group. With each subsequent cycle, two more carbons are added to the backbone chain until elongation is terminated by a thioesterase enzyme.

Even though most of the enzymes responsible for fatty acid biosynthesis have been successfully identified, further studies can power the discovery of new target genes and enzymes associated with fatty acid response mechanisms. The genes and enzymes identified from these studies can be engineered to improve the tolerance of a strain to toxic products, the transport mechanisms of fatty acids, and the specificity to produce fatty acids of the desired chain length. Systems Biology approaches that have been successfully applied for strain improvement and for increased production of different chemicals could potentially meet the needs of obtaining new knowledge about fatty acids and their effect on *E. coli* growth and metabolism.

4.1.2. Overproduction of fatty acids in *Escherichia coli* for chemical and biofuel production

Fatty acids are energy-dense molecules that are produced, at different levels, in every organism, making them a suitable target for biofuel production. In addition, their relatively simple structures make them attractive as building blocks for the chemical industry for the synthesis of polymers and activation with different functional groups. The increasing interest towards alternative sources for production of biorenewable fuels and chemicals, have powered numerous studies with the goal of utilizing the fatty acid biosynthetic cycle for the microbial production of fuels and chemicals. However, further investigation about regulation, productivity and toxicity of fatty acids and their intermediates is still needed in order to achieve industrial-scale production.

First, the use of different thioesterases has facilitated modifications in fatty acid chain-length, degree of unsaturation, and yield (Lu et al., 2008; Zhang et al., 2011). However, expression of thioesterases needs efficient tuning, since too high expression has been shown to impair free fatty acid production (Yu et al., 2011). In addition, high titers of fatty acids in the medium can cause severe defects in cell viability (Desbois and Smith, 2010). Nonetheless, promising results have been obtained from deletion of the fatty

acid transporter FadL and overexpression of *tesA* (Liu et al., 2012). The deletion of genes involved in the β-oxidation cycle has also been investigated as a means to prevent product degradation. Free fatty acid levels have been enhanced by partial deletion of β-oxidation degradation, mainly by the deletion of *fadD* (Steen et al., 2010; Zhang et al., 2011). Deletion of genes responsible for acetate formation has been tested to improve malonyl-CoA and free fatty acid titers (Li et al., 2012; Zha et al., 2009; Zhang et al., 2011). In addition, overexpression of acetyl-CoA carboxylase has also been investigated and, in combination with other genetic modifications, has successfully enhanced free fatty acid production (Davis et al., 2000; Lu et al., 2008). These are just a handful of many valuable studies that have been performed with the goal of increasing fatty acid productivity in *E. coli* by pathway-specific genetic modifications. In addition, modifications to regulators involved in fatty acid synthesis and degradation have also provided beneficial results (Dellomonaco et al., 2010; Zhang et al., 2012). One of the best examples of engineering regulators to achieve efficient fatty acid production in *E. coli* was performed by Dellomonaco, *et al.* (Dellomonaco et al., 2011) with the reversal of the β-oxidation cycle, by introducing mutations in regulatory genes *fadR, crp, arcA*, and *atoC*, combined with overexpression of *fadB*, *fadA*, and *fadM*.

All these studies present promising engineering strategies for efficient production of a wide range of fatty acids from different carbon sources, but

increasing fatty acid titers have been shown to induce stress responses and decrease cell viability (Desbois and Smith, 2010; Lennen et al., 2011; Royce et al., 2013). Additional metabolic engineering strategies to increase tolerance of *E. coli* to fatty acids are much needed and are the motivation of this work.

4.1.2.1. Toxicity Considerations

The presence of short and medium-chain fatty acids in the culture medium affect the growth characteristics of *E. coli* in different ways. Variations in specific growth rate and maximum cell concentration can express the degree of inhibition caused by fatty acids to *E. coli*. The degree of inhibition has been found to be dependent on different parameters such as fatty acid chain-length, culture medium, pH, and the growth stage of the culture used for inoculation. Fay and Farias (Fay and Farias, 1975) studied the inhibitory effect of fatty acids of varying chain-lengths on the growth of *E. coli* K-12, also comparing growth on different media. The doubling time of the cultures was affected by the presence of short chain fatty acids (C_4-C_6) in the medium, showing increased growth inhibition with decrease in chain length. Other studies that analyzed the effect of fatty acids on *E. coli* have reported differences in growth rates (Thompson and Hinton, 1996) and size of the cells (Nakamura and Hase, 1984). A recent study by Nakanishi

(Nakanishi et al., 2009) reported a negative effect to enterohaemorragical *E. coli* (EHEC) at fatty acid concentrations higher than 50mM.

Earlier studies have shown that exposure to FAs affects *E. coli* growth (Desbois and Smith, 2010; Fay and Farias, 1975), suggesting enzyme inhibition, acidification of the cytoplasm, redox imbalances, and cellular membrane damage as potential routes for inhibition (Black and Dirusso, 1994; DiRusso et al., 1999). Recent efforts to microbially produce SCFAs have motivated fundamental studies aimed at identifying the toxicity mechanisms of these molecules in *E. coli.* Most of these have focused on analyzing the effects of FAs on the cellular membrane, studying changes in membrane composition (Lennen and Pfleger, 2013; Royce et al., 2013) and transport proteins (Lennen et al., 2011; Lennen et al., 2013). In order to explore other strategies for improving tolerance, a transcriptome analysis of octanoic acid challenge in *E. coli* was performed by Royce, *et al* (Royce et al., 2014). Even though membrane damage still appears to be the limiting factor in tolerance to SCFAs, their study revealed several differentially expressed genes that suggest intracellular acidification as another mechanism of inhibition. A proteomic analysis of the effect of oleic acid on *E. coli* was performed by Han, *et al* (Han et al., 2008), but their study focused on effect of long-chain FAs and developing an oleic acid-inducible promoter for the production of recombinant proteins, instead of analyzing its toxic effects.

This work covers the first known proteomic analysis to assess the inhibitory effect of SCFAs in *E. coli*. Results obtained from this analysis could potentially benefit future engineering efforts by guiding the design and construction of industrial strains that produce SCFAs with increased tolerance and productivity.

4.2. Materials and Methods

Strains, genetic and analytical methods, and procedures for proteomic analysis are detailed in Chapter 3. This section explains culture medium and conditions that are specific to the current chapter.

4.2.1 Culture Medium and Cultivation Conditions

The minimal medium designed by Neidhardt et al. (Neidhard.Fc et al., 1974), with 40mM MOPS and Na_2HPO_4 in place of K_2HPO_4, supplemented with 5g/L glucose, 10g/L tryptone, 5g/L yeast extract, 1.32mM Na_2HPO_4 was used for all fermentations, unless stated otherwise. Octanoic acid ($C_8H_{16}O_2$) was added in different concentrations (0, 10, 15, 20mM), as stated in each specific case. When octanoic acid was added, pH was adjusted to 7.0 with NaOH. Antibiotics (100μg/mL ampicillin, 34μg/mL chloramphenicol and 50μg/mL kanamycin) and inducers (0.1mM isopropyl β-d-1-thiogalactopyranoside (IPTG), 100ng/mL anhydrotetracycline and 200μM

arabinose) were included when appropriate, unless stated otherwise. All chemicals were obtained from Fisher Scientific Co. (Pittsburgh, PA) and Sigma-Aldrich Co. (St. Louis, MO).

Fermentations were conducted in 250mL Pyrex Erlenmeyer flasks (No.4980, Corning Inc., Corning, NY) filled with 20mL of the above culture medium and sealed with foam plugs filling the necks. Three colonies of the desired strain were cultivated for 3–4h in LB medium with appropriate antibiotics and used as the inoculum for an initial OD of 0.05 for all fermentations. After inoculation, flasks were induced as needed and incubated at 37°C and 140rpm in a Lab Companion SI-600 Benchtop Incubator Shaker (JEIO Tech Co., Seoul, Korea) for 4 hours, unless otherwise stated.

4.2.2. Intracellular pH Assay

Strains carrying the pBad24-TorA-GFPmut3* (pJDT1) plasmid (Thomas et al., 2001) were grown in 250mL Pyrex Erlenmeyer flasks (No.4980, Corning Inc., Corning, NY) filled with 25mL of potassium-modified LB media (LBK) (Maurer et al., 2005) containing 100μg/mL ampicillin and 200μM arabinose at 37ºC and 140rpm in a Lab Companion SI-600 Benchtop Incubator Shaker (JEIO Tech Co., Seoul, Korea) until mid-exponential phase ($OD_{600} \sim 0.5$). The cells were harvested at 5000g and 4ºC for 20 minutes and

resuspended in phosphate-buffered saline (PBS) (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4). Samples were subjected to different concentrations of octanoic acid (0, 10, 15, and 20mM) and pH was adjusted to 7.0. Intracellular pH for each sample was calculated using a calibration curve generated with sodium benzoate, according to (Royce et al., 2014; Wilks and Slonczewski, 2007). Fluorescence intensity was measured in a BioTek Synergy HT Micro-plate reader (BioTek, Winooski, VT), adding 100µL of samples to opaque bottom Costar 96-well plates (Corning Inc, Corning, NY). The plates were analyzed at 30ºC, using an excitation filter of 485/20nm, and an emission filter of 528/20nm.

4.3. Results

4.3.1. Inhibitory effects of exogenous octanoic acid in *E. coli*

The inhibitory effects of FAs on *E. coli* have been studied in various occasions using different FA chain-lengths and under different culture conditions (Hassinen et al., 1951; Marounek et al., 2003; Royce et al., 2013; Sprong et al., 2001). The degree of inhibition has been found to be dependent on different parameters, such as FA chain-length, culture medium, pH, and growth stage of the culture used for inoculation (Fay and Farias, 1975; Nakamura and Hase, 1984; Thompson and Hinton, 1996). In this study, octanoic acid toxicity was analyzed in order to determine the extent of

inhibition and to select the optimum comparative conditions for the proteomic analysis. *E. coli* was grown with increasing concentrations of exogenous octanoic acid, ranging from 0mM (control) to 40mM (Figure 4.2). Growth of wild-type strain MG1655 in the absence of octanoic acid reached an optical density (OD) of 4.2 after 4 hours, but as octanoic acid concentration was increased, the maximum OD reached by the cultures decreased by 32, 48, 92, and 100% for cultures with 10, 15, 20, and 30mM, respectively. Under the analyzed conditions, 30mM was the lowest concentration at which *E. coli* MG1655 was not able to grow. This was designated as the minimum inhibitory concentration (MIC) for the conditions used in this study. Based on this fact, the concentration that is 50% of the minimum inhibitory concentration (MIC_{50}), 15mM, was selected for the comparative proteomic analysis.

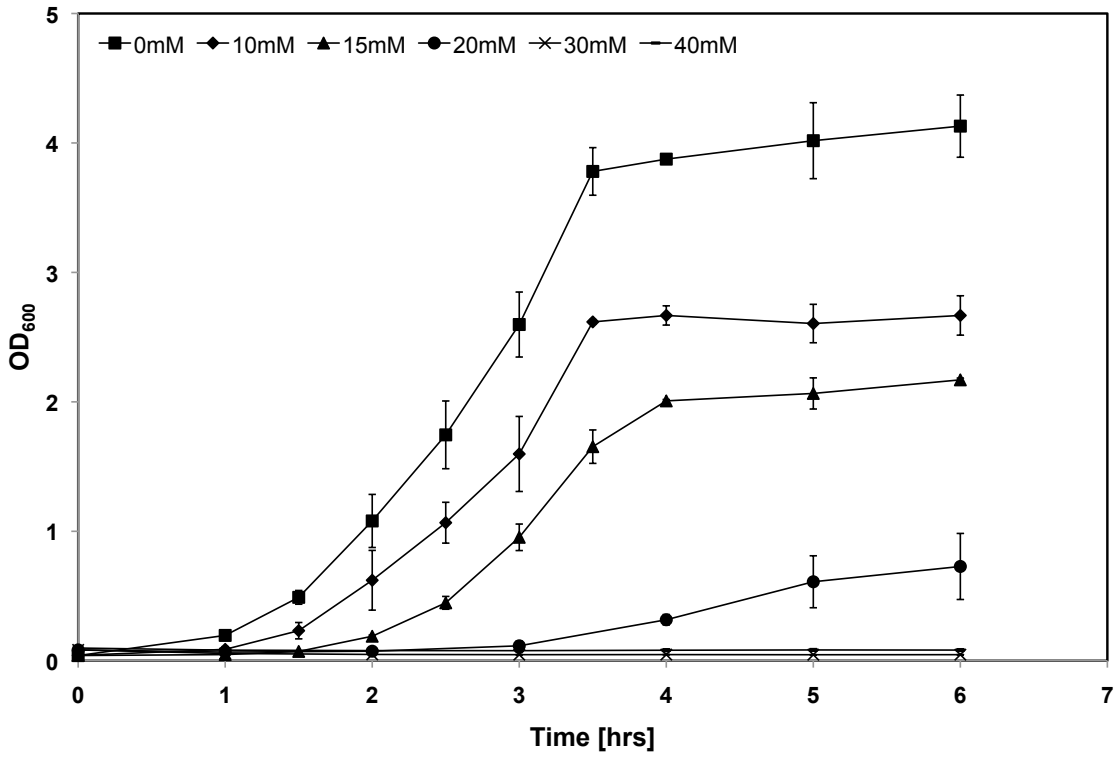


Figure 4.2. Growth curves of wild-type *E. coli* MG1655 under different concentrations of octanoic acid (C8) show inhibitory effects of exogenous FAs. Longer lag phases and lower maximum ODs were obtained with increasing concentrations of C8. No growth was observed at concentrations equal or higher than 30mM.

The above analysis showed that exogenous octanoic acid stress led to an extended lag phase and inhibited maximum cell growth, while the cell growth rates showed a slight decrease with increasing concentration beyond 10mM: 0.968, 1.121, 0.948, and 0.897hr^{-1} for 0, 10, 15, and 20mM, respectively (Figure 4.2). Fay and Farias also studied the inhibitory effects of FAs of varying chain-length on growth of *E. coli* K-12, comparing growth on different media (Fay and Farias, 1975). The doubling times in their cultures were affected by the presence of SCFAs (C_4-C_6) in the medium, showing

increased growth inhibition with shorter chain-lengths. Other studies have reported differences in growth rates (Thompson and Hinton, 1996) and cell size (Nakamura and Hase, 1984). Additional studies have shown that carboxylic acid toxicity increases at lower pH values, as the media pH nears the pK_a (Liu et al., 2013; Royce et al., 2013; Stratford and Anslow, 1996). Similar studies have been performed with short- and medium-chain fatty acids in the yeast *Saccharomyces cerevisiae* (Liu et al., 2013; Stratford and Anslow, 1996). These studies also showed longer lag phases with increasing concentrations of decanoic acid (Stratford and Anslow, 1996) and decreasing specific growth rates with increasing concentrations of hexanoic, octanoic, and decanoic acid (Liu et al., 2013), although at a higher degree than in *E. coli*.

4.3.2. Proteomic analysis of *E. coli* under exogenous octanoic acid stress

Previous studies have shown that systems biology approaches can provide valuable information about production and inhibition mechanisms of molecules and can power engineering strategies to improve tolerance and overall productivity in microbial fermentations (Jarboe et al., 2011; Rodriguez-Moya, 2010). We performed a proteomic analysis of wild-type *E. coli* MG1655 during mid-exponential growth (OD_{600} between 0.7-0.8) in LB-MOPS media with 5% glucose at pH 7.0, under the two culture conditions selected from the toxicity analysis in the previous section, 0mM and 15mM C8

(MIC_{50}). Three biological replicates of each condition were analyzed by 2D gel electrophoresis and were compared using Progenesis Same Spots software, as explained in the Materials and Methods chapter (Chapter 3). This software normalized individual spot intensities using the total spot intensity per gel to compensate for variations between gel replicates. The log of each normalized value was then calculated to reduce spot volume to spot deviation dependency and a statistical comparison of the relative spot volumes using ANOVA was performed. Spots with p-values lower than 0.05 were considered statistically significant. Following this analysis, out of 1,010 total protein spots, 33 spots had p-value $\leq$ 0.05, of which 24 had an abundance ratio of more than 1.5 fold (Figure 4.3, Table 4.1).

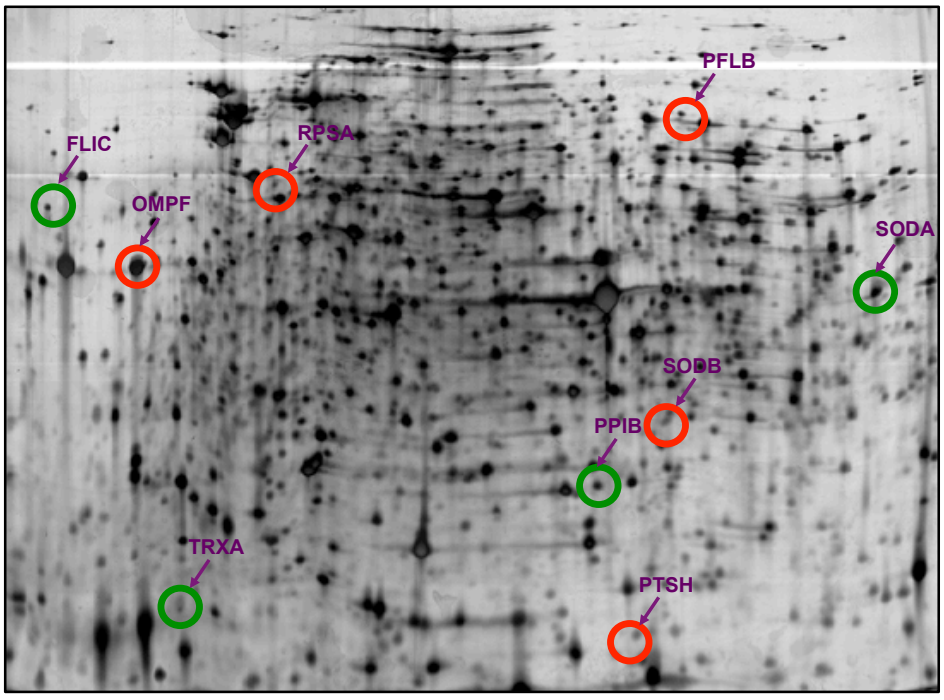


Figure 4.3. Differentially expressed proteins of wild-type *E. coli* MG1655 exposed to 15mM octanoic acid (C8) until mid-exponential phase. Circles

represent proteins with an abundance fold-difference higher than 1.5 (Red, decreased abundance; Green, increased abundance). Gel corresponds to 0mM condition.

Table 4.1. Differentially expressed proteins under octanoic acid stress

Spot #	Protein Name (*gene*)	Protein Function	Differential Expression Fold	p-value	GI number
3	Pyruvate formate lyase (*pflB*)	Formate + Ac-CoA ↔ Pyruvate + CoenzymeA	-4.3	0.004	218704330
4	Mn-superoxide dismutase (*sodA*)	Response to oxidative stress	+3.8	0.022	13399622
5	Hpr (*ptsH*)	Non-specific sugar transport, PTS system	-2.9	0.008	157832561
11	Flagellin (*fliC*)	Flagellar structure and motility	+2.3	0.018	16129870
15	Oxidized thioredoxin (*trxA*)	Reduction of cytoplasmic enzymes, trans-membrane transfer of reducing potential	+1.9	0.006	68304124
18	Ribosomal protein S1 (*rpsA*)	Protein synthesis (mRNA translation)	-1.7	0.002	15800772
20	Outer membrane porin F (*ompF*)	Transport of sugars, ions, amino acids, and medium and long-chain FAs	-1.6	0.018	157831181
21	Fe-superoxide dismutase (*sodB*)	Response to oxidative stress	-1.6	0.029	93278547
22	Rotamase B (*ppiB*)	Protein folding	+1.5	0.039	16128509

Spot #	Differential Expression Fold	p-value	Spot #	Differential Expression Fold	p-value
1	-6.4	0.046	19	+1.7	0.026
2	+4.5	0.031	23	+1.5	0.003
6	+2.9	0.045	24	+1.5	0.019
7	-2.7	0.047	25	+1.4	0.041
8	-2.7	0.044	26	-1.4	0.003
9	-2.4	0.025	27	-1.4	0.047
10	+2.3	0.028	28	+1.3	0.013
12	-2.3	0.026	29	+1.3	0.022
13	-2.0	0.031	30	+1.3	0.010
14	+1.9	0.013	31	+1.3	0.012
16	-1.8	0.018	32	+1.2	0.034
17	-1.8	0.018	33	+1.2	0.003

Eight of the differentially expressed spots were subjected to MALDI-TOF/MS analysis, followed by homology search using MASCOT and identification from the NCBI database. Another spot with differential abundance was identified by matching to *E. coli* master gels from the ExPASy online database (Gasteiger et al., 2003; Wilkins et al., 1999). The nine proteins those identified as differentially expressed under octanoic acid stress (Table 4.1) belong to four main categories that are hypothesized to have important roles on the tolerance of *E. coli* to fatty acids: (1) transport and structural (OmpF, HPr, and FliC), (2) oxidative stress (SodA, SodB and TrxA), (3) metabolic functions (HPr, PflB), and (4) protein synthesis (PPiB and RpsA). A more detailed examination of the roles of these proteins on the response of *E. coli* to SCFAs is presented in the following sections.

4.3.3. Do differentially expressed proteins play a role in the response of *E. coli* to octanoic acid?

As an initial approach to assess the potential role of the differentially expressed proteins on the tolerance of *E. coli* to fatty acids, the effect of deletion and overexpression mutants for each of the proteins on growth with FAs was analyzed. Single-gene deletion mutants from the Keio collection were used to assess the absence of these genes (Baba et al., 2006). Even though the *E. coli* base strain for the Keio collection (BW25113) is different than that

used for the initial toxicity analysis (MG1655), they were found to have similar response to octanoic acid (data not shown). In order to assess overexpression of the differentially expressed proteins, high-copy plasmids expressing individual genes were extracted from ASKA collection mutants (Kitagawa et al., 2005) and transformed into the *E. coli* strain BW25113. Genetic modifications were identified as beneficial or detrimental by calculating a difference of maximum growth at 4 hours of culture, [OD $\text{Control}_{(0\text{mM C8})}$ – OD $\text{Stress}_{(15\text{mM C8})}$]. Following this approach, mutant strains that exhibit a significant difference in growth with respect to the wild-type indicate a strong influence of that particular gene or protein on response to the presence of FAs. The differences in maximum growth for deletion and overexpression mutants are shown in Figure 4.4.a and 4.4.b, respectively.

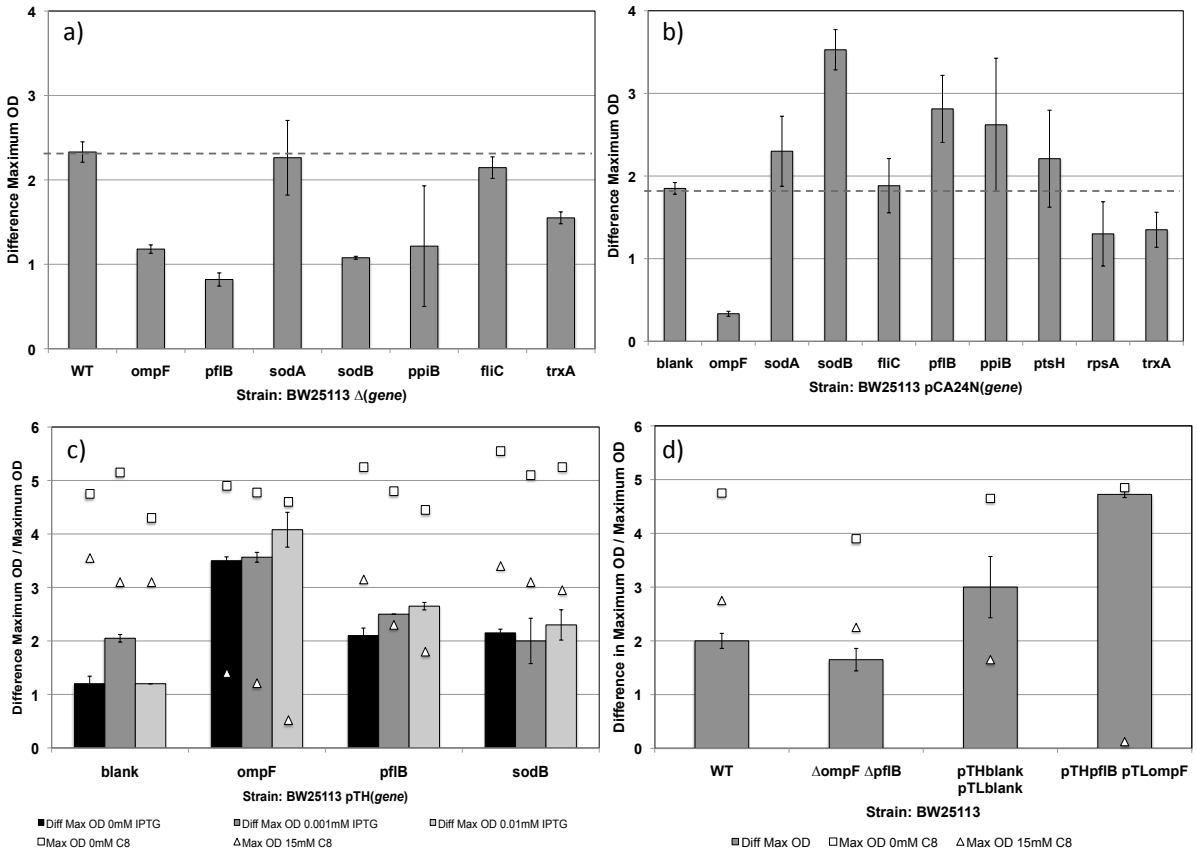


Figure 4.4. Differences of maximum OD for mutants of differentially expressed proteins under C8 stress. Differences in maximum OD for (a) deletion and (b) high-overexpression mutants. (c) Differences of maximum OD for *ompF, sodB,* and *pflB* over-expression mutants in pTH_A vector under different concentrations of C8 and varying inducer (IPTG) concentrations. (d) Differences of maximum OD for *ompF* and *pflB* double mutants. OD was measured at 4hrs of culture in all cases. Difference of maximum OD = $[OD_{0mMC8} - OD_{15mMC8}]$. (□, OD_{0mMC8}; Δ, OD_{15mMC8}).

Deletion mutants for *sodA* and *fliC* did not lead to any advantage or disadvantage over the wild-type. On the other hand, deletion of genes *ompF, sodB, pflB, ppiB,* and *trxA* showed small differences between growth in absence and presence of FAs when compared to the wild-type, suggesting less growth impairment in the presence of FAs. Of special interest is the advantage conferred by deletion of OmpF, since this porin has been suggested

to participate in FA transport across the outer membrane (Nunn et al., 1986; van den Berg, 2010), and deletion of the protein could be partially impeding FAs from entering the cell. It is important to mention that deletion mutants for *rpsA* could not be obtained since it is an essential gene for *E. coli* growth. Also, the Δ*ptsH* mutant did not grow under the conditions used for this study.

As can be observed in Figure 4.4.b, overexpression of *sodA, fliC,* and *ptsH* did not confer significant advantages or disadvantages over the wild-type. Overexpression mutants for *sodB, pflB,* and *ppiB* exhibited the greatest differences in growth, showing that overexpression of these individual genes affect growth of *E. coli* under FA stress. In this case, mutants with small differences were due to low growth under both conditions (0 and 15mM C8), possibly due to accumulation of toxic levels of protein or, in the case of OmpF, possible membrane disruption.

Based on the above deletion and overexpression analyses, *ompF, sodB,* and *pflB* were selected as the genes exhibiting most impact on *E. coli* growth in the presence of FAs by following a pattern of exhibiting a smaller growth difference when deleted and a larger growth difference when overexpressed. This pattern was set as the rational threshold for narrowing potential targets for further analyses. It is notable that OmpF is an exception to this pattern, since it exhibited smaller differences in both cases, deletion and overexpression. Still, as mentioned above, OmpF is of special interest because

it is a potential SCFA transporter and could also directly affect membrane integrity.

In order to eliminate potential toxicity associated with excessive overexpression from ASKA collection vectors, overexpression of these three genes was analyzed in the vector pTrcHis2A, a lower-copy vector, and under a range of inducer (IPTG) concentrations. Figure 4.4.c shows differences in maximum growth for *ompF, pflB,* and *sodB* when overexpressed from the pTH_A vector in the BW25113 background. The difference in maximum growth for pTH_A*sodB* was double that of the control strain expressing the blank plasmid and its growth under octanoic acid stress is only slightly lower than that of the control strain. Still, this change does not seem to be solely caused by FA exposure, since growth of both strains in the 0mM condition was slightly higher than the wild-type. On the other hand, strains expressing pTH_A*ompF* and pTH_A*pflB* grow at the same levels than the wild-type in the 0mM condition, but their tolerance to octanoic acid noticeably decreases with increasing levels of expression (increasing IPTG concentration). Again, *ompF* and *pflB* stand out for exhibiting the largest impact on growth under FA stress. Their combined effect on *E. coli* growth was then studied using a double deletion mutant and double overexpression strain in the BW25113 background (Figure 4.4.d). For the double overexpression mutant, *ompF* was expressed in vector pTL (Blankschien et al., 2010), a vector with a similar

copy number than that of pTH_A, but a different antibiotic marker (kan^R, instead of amp^R). Although growth of the Δ*ompF* Δ*pflB* mutant in 15mM did not increase when compared to the wild-type, not showing improved tolerance, it is still the closest to the wild-type under the same conditions, and shows the smallest difference in maximum growth. Moreover, the detrimental effects of the pTH_A*pflB* pTL*ompF* strain confirm that these two genes play key roles in the response of *E. coli* to octanoic acid. The individual effect of *ompF, pflB,* and *sodB* were also analyzed using the low-copy vector pZS (Yazdani and Gonzalez, 2008) under different inducer (anhydrotetracycline) concentrations, but the expression level was possibly too low and no differences in growth between the mutants were observed (data not shown).

4.3.4. Exposure to octanoic acid leads to differences in intracellular pH between OmpF mutants and wild-type cells

In order to further investigate the effect of OmpF on the response mechanism of *E. coli* to FAs, the effects of different *ompF* modifications on intracellular pH were analyzed. It is known that exposure to octanoic acid lowers intracellular pH (Royce et al., 2014), and that disturbances to the pH gradient across the membrane disrupt nutrient transport (Bassilana et al., 1984; Leblanc et al., 1980). Therefore, strategies to overcome this acidification effect are needed to maintain cell viability. A pH-dependent

green fluorescence protein (Wilks and Slonczewski, 2007) was used to quantify intracellular pH of the strains under different concentrations of octanoic acid at neutral buffer pH. The difference in intracellular pH for two conditions, 0 and 15mM C8, was calculated for each of the strains (Figure 4.5). The difference in intracellular pH for the *ompF* deletion strain was smaller than the difference for the wild-type, suggesting that deletion of OmpF could be partially impeding the entrance of FAs into the cell, therefore minimizing changes in intracellular pH. This behavior can also be observed when comparing strains expressing pTL*ompF*, with and without the gene deletion. The strain with lower OmpF expression, i.e. Δ*ompF* pTL*ompF,* exhibited a smaller difference in intracellular pH, although statistically the changes do not appear to be significance.

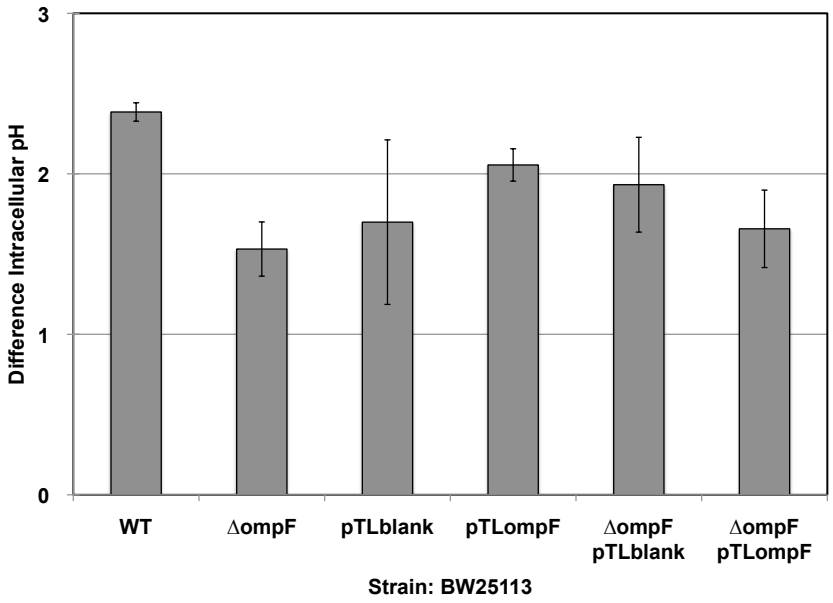


Figure 4.5. Differences of intracellular pH for *ompF* mutants under octanoic acid (C8) stress. Difference of intracellular pH = [Intracellular pH_{0mMC8} – Intracellular pH_{15mMC8}].

4.3.5. Changes in expression of other outer membrane proteins in response to the presence of octanoic acid

The cellular membrane has been presented as one of the main targets in the mechanisms of microbial inhibition by carboxylic acids (Desbois and Smith, 2010; Ricke, 2003). Damages to the cellular membrane of *E. coli* (Lennen et al., 2011; Royce et al., 2013) and yeast (Liu et al., 2013) by exposure to FAs have recently been reported. These studies have presented crucial insights about membrane composition, fluidity, and integrity. Lennen, *et al* (Lennen and Pfleger, 2013), reported improved cell viability after restoring normal FA membrane content. Still FA titers have not been

improved and further investigation is needed. The proteomic analysis performed in this study is in agreement with previous reports about membrane damage under FA stress. As presented in section 4.3.3, changes in the membrane are implied from the differential expression of the outer membrane protein, OmpF, and of proteins affected by oxidative stress, which is one of the main effects of membrane damage.

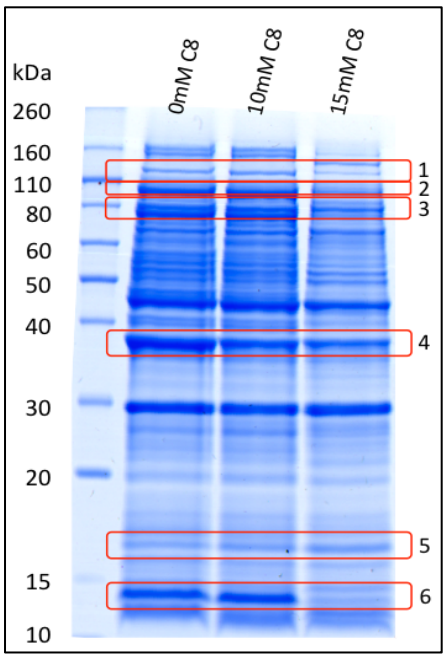


Figure 4.6. SDS-PAGE of purified membrane proteins. Comparison of protein expression with increasing octanoic acid (C8) concentration (0, 10 and 15mM).

Additional membrane proteins could have been suppressed from the 2D gel electrophoresis analysis due to fractionation of membrane and cytoplasmic proteins during clean up procedures. Therefore, expression of membrane proteins was studied in more detail by purifying the insoluble protein fraction and analyzing this fraction via SDS-PAGE (Figure 4.6, Table

4.2). In this analysis, protein bands with changes in intensity were selected by visual inspection, since these changes were evident, and a fold difference was not calculated. Protein bands were identified by MALDI-TOF/MS, as explained in the Materials and Methods chapter (Chapter 3). As anticipated, other outer membrane proteins, OmpX, and the lipoprotein Slp, were differentially expressed under octanoic acid stress. In addition, proteins AceE and FrdA, involved in metabolic functions, and the phosphoprotein TypA, were also differentially expressed.

Table 4.2. Differentially expressed membrane proteins

Spot #	Protein Name	Protein Function	GI number
1	Pyruvate dehydrogenase, subunit of E1p component (*aceE*)	Pyruvate + Coenzyme A + $NAD^+ \leftrightarrow$ Acetyl-CoA + CO_2 + NADH	545157572
2	GTP-binding protein (*typA*)	Possibly involved in ribosome structure or function	485952772
3	Fumarate reductase, flavoprotein (*frdA*)	Fumarate + Menaquinol → Menaquinone + Succinate	487568364
4	Outer membrane porin C (*ompF*)	Export of extracellular proteins, resistance to antibiotics	157831181
5	Outer membrane lipoprotein (*slp*)	Starvation protein	485732731
6	Outer membrane protein X (*ompX*)	Acid-induced protein, affected by medium osmolarity	6435772

4.3.6. Translating effects to hexanoic acid: response of *ompF* and *pflB* mutants

In an effort to extrapolate the findings of the response to octanoic acid to other SCFAs, the modifications of the two key genes, *ompF* and *pflB,* were also analyzed in growth of *E. coli* under hexanoic acid (C6) stress. Maximum growth and differences in maximum OD for these strains are shown in Figure

4.7. In this case, the beneficial effects of proteins OmpF and PflB for FA tolerance are even more evident. Both overexpression mutants exhibited larger differences than the control strain expressing the blank plasmids, while both deletion mutants exhibited smaller differences of maximum OD than the wild-type. Overexpression of *ompF* had a larger negative effect on growth under FA stress than *pflB* and, therefore, a larger difference of maximum OD. The most interesting result in this analysis relates to the growth of the Δ*ompF* mutant in the presence of hexanoic acid. Although deletion of *ompF* resulted in decreased maximum growth at 0mM hexanoic acid in comparison to the wild-type, the mutation enabled growth slightly higher than the wild-type under hexanoic acid stress. These are very promising results since, in addition to confirming *ompF* as a key target for FA tolerance, it suggests that the insights gained from the analysis with octanoic acid may be potentially translated to other FAs of similar chain-length with comparable results.

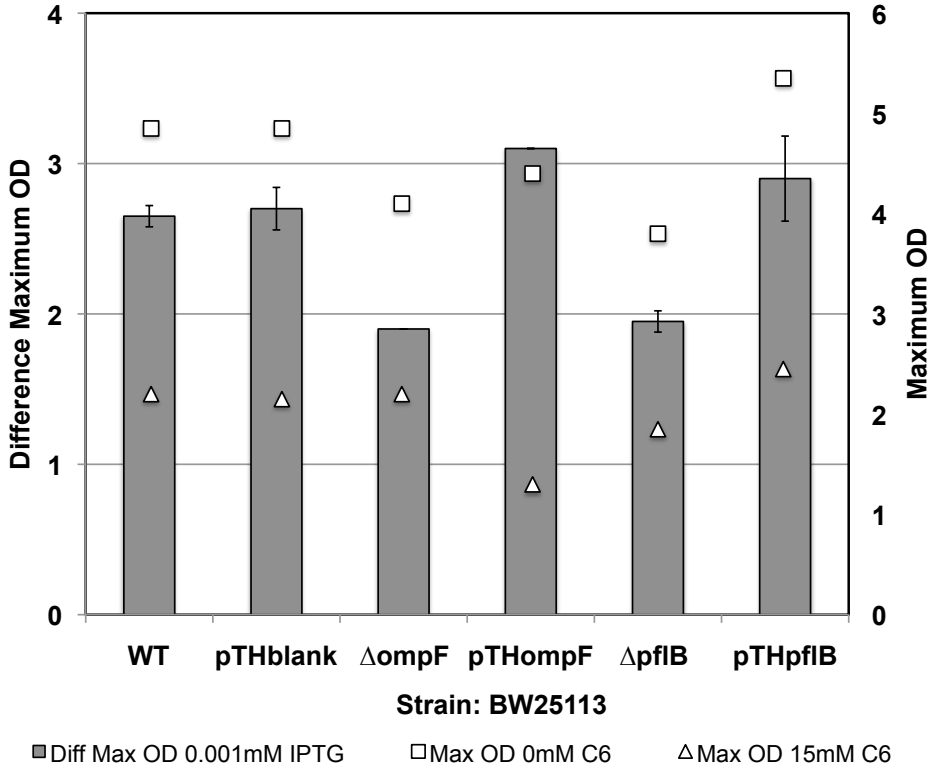


Figure 4.7. Differences of maximum OD for *ompF* and *pflB* mutants under hexanoic acid (C6) stress. OD was measured at 4hrs of culture in all cases. Difference of maximum OD = [$OD_{0mMC6} - OD_{15mMC6}$]. (□, OD_{0mMC6}; Δ, OD_{15mMC6}).

4.4. Discussion

Although there is no doubt that cells use different response systems to cope with the presence of SCFAs, the combined perturbation of proteins from the four groups affected by FA stress in this study (transport and structure, oxidative stress, metabolic functions, and protein synthesis) suggests a mechanism of inhibition mainly concentrated in the cellular membrane. Changes in proteins responsible for trans-membrane transport and membrane structure directly affect membrane integrity. In addition,

oxidative stress, demonstrated by differential expression of superoxide dismutases, can be caused by damage to the electron transport chain in the cell membrane. A combination of membrane damage and oxidative stress will also cause imbalances of metabolites in the cytoplasm, resulting in changes to metabolic functions in the cell. A more detailed explanation of these effects is presented below.

Several outer membrane proteins involved in the transport of metabolites into and out of the cell were prominent in this study. The outer membrane porins OmpF and OmpX, and the lipoprotein Slp were differentially expressed under octanoic acid stress. These three proteins have been previously reported to respond to acid stresses, including exposure to nalidixic acid (Lin et al., 2012) and FAs (Lennen et al., 2011; Royce et al., 2014; Tucker et al., 2002). In a recent study, altered tolerance of *E. coli* to longer-chain FAs was achieved by modifying membrane lipid composition (Lennen and Pfleger, 2013), indicating an important role of this membrane property on the response mechanism to FAs. Moreover, changes in membrane lipid composition have been associated with changes in the synthesis and assembly of outer membrane proteins (Bocquetpages et al., 1981; Ried et al., 1990). These effects are potentially extended to shorter-chain FAs, as evidenced by differential expression of OmpF, OmpX, and Slp in this study. In addition, OmpF, one of the two most important outer

membrane porins in *E. coli* and responsible for the transport of many metabolites through the outer membrane (Hoenger et al., 1993), was hypothesized in early studies as a transporter of medium- and long-chain FAs through the outer membrane (Nunn et al., 1986; van den Berg, 2010). However, further characterization of outer membrane porins and FA transport revealed that long-chain FAs do not use porins to traverse the outer membrane, since porin structure and residue configurations inside their channels make it energetically unfavorable for hydrophobic molecules to enter and traverse the channels (Schulz, 2002). FadL, an outer membrane protein involved in long-chain FA transport, remained as the only known channel with an established role in uptake of hydrophobic compounds (Black, 1990; Black et al., 1987), but additional studies revealed that Δ*fadL* mutants were able to grow on medium-chain FAs, indicating that these more polar compounds can enter *E. coli* via other ways, presumably porin channels (Black and DiRusso, 2003; Black et al., 1987). Even though short-chain FAs can enter the cell by free diffusion, the availability of a channel like OmpF can facilitate their transport into the cell. Therefore, the decreased expression of OmpF found in this study can be a mechanism to prevent FAs from entering *E. coli* and disrupting intracellular pH and oxidative balance (Figure 4.8).

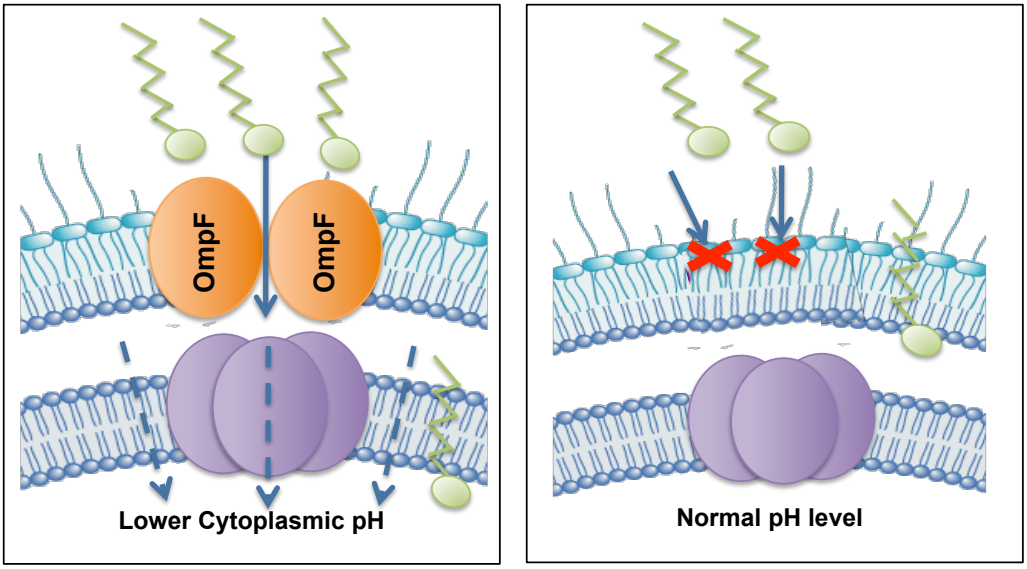


Figure 4.8. Schematic of the proposed role of OmpF in the response mechanism of *E. coli* to SCFAs. OmpF is believed to facilitate SCFA transport into the cells, allowing them to disrupt intracellular pH and oxidative balances (left). Deletion of OmpF may reduce entrance of SCFAs into the cells and, therefore, maintain pH closer to normal (right).

In addition to the aforementioned outer membrane proteins, HPr and FliC were two other differentially expressed proteins with transport and structural functions. HPr is a component of the phosphotransferase system (PTS) in *E. coli.* This cytoplasmic protein, in conjunction with Enzyme I, is required for transport and phosphorylation of all PTS sugars (Postma et al., 1993; Saffen et al., 1987). Since OmpF is one of the outer membrane porins through which carbohydrates can enter the cell (Gosset, 2005), its decreased expression can reflect a decrease in intracellular concentrations of carbohydrates and, in turn, decreased expression of HPr. Although it has been

mentioned that membrane changes can disturb intracellular metabolic processes by affecting metabolite exchange into and out of the cell, the role of HPr on tolerance to fatty acids may be more clear in the context of other differentially expressed proteins related to metabolic functions. On the other hand, FliC, or flagellin, is a protein responsible of forming the flagellar structure in *E. coli* (Lehti et al., 2012). Flagella are important for survival since they enable cellular movement towards favorable or away from detrimental conditions in the growth media, but, at the same time, they present an energy burden to the cell when activated (Lehti et al., 2012). Due to this burden, their expression is tightly regulated and they are activated in response to stresses, such as pH (Maurer et al., 2005) and exposure to different antibacterial agents (Lenahan et al., 2014; Sheridan et al., 2013; Tobe et al., 2011). Differences in expression of FliC and flagellar length have been reported in the response of enterohemorrhagic *E. coli* to triclosan and other antimicrobial agents, but increases in motility was not observed (Lenahan et al., 2014; Sheridan et al., 2013). The role of flagella in the response mechanism of *E. coli* to FAs is unclear, but its increased expression could be due to extracellular stresses from exposure to exogenous FAs, and may not be translated to the effects of FAs produced intracellularly. This is further supported by the omic study reported by Lennen, *et al* (Lennen et al., 2011), in which the analysis of

an FA-producing strain did not evidence changes to flagellar genes or proteins.

Oxidative stresses, demonstrated by differential expression of several proteins in this study, can be caused by damage to the electron transport chain in the cell membrane. As mentioned above, differences in expression of SodA, SodB and TrxA suggest that the presence of FAs affects redox balance in the cells. Superoxide dismutases (SODs) are metalloenzymes that catalyze the conversion of the superoxide anion to hydrogen peroxide and dioxygen (McCord and Fridovic.I, 1969). The Mn- (SodA) and Fe-SODs (SodB) in *E. coli* are highly homologous (Fink and Scandalios, 2002) and both are equally important in protection against oxygen toxicity (Schellhorn and Hassan, 1988). Even though both SODs have been reported as major contributors to acid tolerance in bacteria, studies have shown a greater role of Mn-SOD than Fe-SOD in response to organic acids (Bruno-Barcena et al., 2010). Transcriptome studies on *E. coli* have also noted activation of oxidative stress response genes under FA exposure (Buzzai et al., 2005; Lennen et al., 2011). Similar studies in *S. cerevisiae* have reported an increase in reactive oxygen species (ROS) and differential expression of superoxide dismutases (Cipak et al., 2008; Legras et al., 2010). The expression of thioredoxin TrxA, an electron donor for ribonucleotide reductase, was also increased in this study, adding to the hypothesis that redox balance is greatly affected by the presence of

fatty acids. Although reports have suggested that TrxA is not affected by the presence of hydrogen peroxide, its function in the transfer of reducing potential could be affected by changes in the oxidative state of the cell (Carmel-Harel and Storz, 2000; Li et al., 2003). Oxidative stress has been linked numerous times to membrane damage on systems under stress to toxic molecules (Lennen et al., 2011; Segura et al., 2012).

A combination of membrane damage and oxidative stress will cause imbalances of metabolites in the cytoplasm, resulting in impaired cellular metabolic functions. Differential expression of two proteins involved in metabolic functions (HPr and PflB) reflects impacts of FAs on vital cellular processes. These proteins are involved in glycolytic reactions around the pyruvate node leading to the production of acetyl-coA. While HPr catalyzes reactions that convert phosphoenolpyruvate to pyruvate (Chandran and Luisi, 2006; Escalante et al., 2012), pyruvate formate lyase participates in catabolism of pyruvate into smaller glycolytic products. In addition, proteins AceE and FrdA, which are involved in pyruvate and fumarate catabolism, respectively, were also differentially expressed in the SDS-PAGE of membrane proteins. Changes in oxidative stress are, once more, evidenced in the differential expression of these proteins. Disturbances in this important node of the glycolytic pathway may be consequence of the disruption of redox balance, as explained above.

Proteins involved in protein synthesis and folding processes were also differentially expressed in the analyses performed in this study. Although protein synthesis and folding involve complex networks of proteins and regulators and its study towards improved tolerance may seem very vast, the differential expression of proteins PpiB, RpsA and the ribosome-binding GTPase TypA is very likely another reflection of oxidative stress in the cell. PpiB is a peptidyl-prolyl *cis-trans* isomerase that has been reported as a catalyst of oxidative protein folding (Schonbrunner and Schmid, 1992). In addition, a recent study about FA utilization in *E. coli* has reported that a mutant RpsA played an important role in decreasing intracellular levels of hydrogen peroxide, which were found to be higher in the FA-utilizing strain in comparison to a glucose-utilizing strain (Doi et al., 2014). Lastly, a *typA* gene homologous to the *E. coli* gene was identified in the chloroplast of the halophytic plant *Suaeda salsa* (Krishnan and Flower, 2008; Wang et al., 2008). Expression of this gene, SsTypA1, in tobacco plants significantly increased tolerance to oxidative stress and reduced H_2O_2 content (Wang et al., 2008). These studies establish the participation of the three proteins in oxidation processes that would be affected by membrane and oxidative stresses in the presence of FAs. As stated before, changes to the cellular membrane can disrupt intracellular pH and oxidative balances creating disturbances to oxidation processes in central metabolism and protein synthesis.

4.5. Conclusions

The findings reported in this work confirm the advantages of omic analyses on guiding strategies to address toxicity challenges in microorganisms. The comparative proteomic analysis of wild-type *E. coli* MG1655 under octanoic acid (C8) stress (15mM) and its reference condition (0mM) revealed a total of 33 protein spots with significant differential expression, nine of which were identified as involved in transport and structural roles (OmpF, HPr, and FliC), oxidative stress (SodA, SodB,a nd TrxA), protein synthesis (PpiB and RpsA) and metabolic functions (HPr and PflB). Additional assays involving deletion and overexpression mutants suggested that membrane damage and oxidative stress are the main routes of inhibition by SCFAs. Furthermore, the differential expression of additional membrane proteins (AceE, TypA, FrdA, Slp, and OmpX) detected by SDS-PAGE reinforced the hypothesis of a strong membrane involvement in the response of *E. coli* to SCFAs. From among these proteins, the outer membrane porin OmpF had the greatest impact on SCFA tolerance. One area that has not yet been exploited for tolerance of SCFAs is the study of FA exporters in *E. coli.* In this context, outer membrane porins that have stood out in this study should be further investigated, and the potential of engineering these targets at the protein structure level should be analyzed.

Chapter 5

Proteomic Analysis of the Fermentative Metabolism of Glycerol in *Escherichia coli*

5.1. Introduction: The potential of glycerol as a feedstock

The surge of biorefineries and renewable products technologies that has been observed in recent years must be accompanied by the continued expansion of new products and feedstocks in order to maintain long-term feasibility and sustainability. Although the industrial-scale production of biofuels and biorenewable chemicals has been diversifying from the long-established bioethanol production with the introduction of chemicals such as 1,4-butanediol (Genomatica, 2013) and biobutanol (www.butamax.com) (Hess, 2006), there is still a need to diversify from traditional carbohydrate feedstocks, which can have high costs arising from pre-conversion treatments or from their essential involvement in the food-feed chain. The search for

new feedstocks from alternative sources, such as by-product or waste streams from industrial processes is now crucial in order to maintain a viable industry.

Glycerol has recently become an inexpensive and abundant source of carbon, resulting as a byproduct of biodiesel, bioethanol, and oleo-chemical production processes. In addition, glycerol can be found at high concentrations in certain species of algae (Oren, 2005). Its accessibility, as well as its high degree of reduction, make glycerol a great alternative for the microbial production of reduced chemicals. The degree of reduction (κ) is a measure of the number of available electrons per carbon atom (Nielsen et al., 2003). The high degree of reduction of carbon atoms in glycerol (κ =4.7) provides a clear advantage over more oxidized carbohydrate-based feedstocks, such as glucose (κ =4) and xylose (κ=4), for the production of fuels and chemicals. However, highly reduced glycerol is difficult to metabolize by microorganisms without an external electron acceptor (i.e., fermentative conditions). In order to make this a viable process, substantial work has been done in the past decade to understand and characterize glycerol fermentative pathways in *E. coli*.

5.1.1. Fermentative metabolism of glycerol in Escherichia coli: 1,2-Propanediol (1,2-PDO)-Ethanol-dependent Model

The metabolic capability of glycerol fermentation is essential to exploit its reduced nature. Although glycerol metabolism was long thought to require the presence of external electron acceptors (Booth, 2005; Quastel and Stephenson, 1925; Quastel et al., 1925), recent efforts have unveiled and characterized several glycerol fermentative pathways.

It was previously thought that glycerol utilization in the absence of an external electron acceptor was constrained to the ability to produce 1,3-PDO, but recent efforts have reported fermentative utilization of glycerol in *E. coli* (Dharmadi et al., 2006) and *Paenibacillus macerans* (Gupta et al., 2009). Even though these microorganisms do not have the ability to produce 1,3-PDO, they can utilize glycerol in the absence of an external electron acceptor by co-producing 1,2-PDO and ethanol as a means of facilitating redox balance and ATP generation, respectively (Figure 5.1) (Gonzalez et al., 2008; Gupta et al., 2009). This model, which was characterized in our lab, consists of an oxidative branch in which glycerol is converted into the glycolytic intermdiate DHAP, by the action of a type II glycerol dehydrogenase (glyDH-II) and a PEP- or ATP-dependent DHA kinase (DHAK), and a reductive branch, in which DHAP is converted to 1,2-PDO. In this model, the DHAP node is of particular

importance, since it enables the reductive branch and ethanol synthesis, both needed for fermentative utilization of glycerol in these organisms.

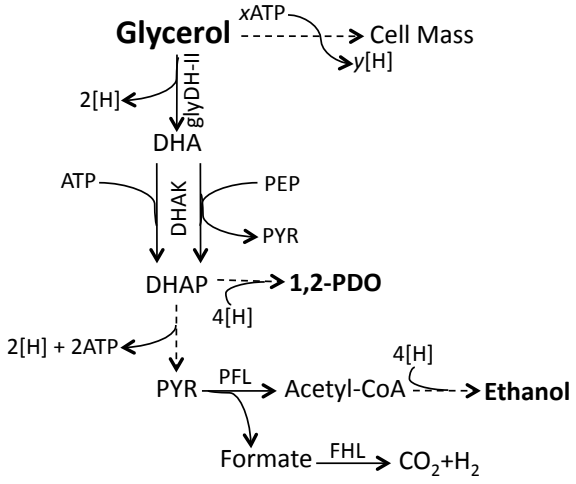


Figure 5.1. 1,2-PDO-ethanol dependent model for fermentative utilization of glycerol in microorganisms. Broken lines represent multiple steps. Relevant reactions are represented by the enzyme names. Abbreviations: DHA, dihydroxyacetone; glyDH-II, glycerol dehydrogenase type II; DHAP, DHA phosphate; DHAK, DHA kinase; PEP, phosphoenolpyruvate; PYR, pyruvate; 1,2-PDO, 1,2-propanediol; PFL, pyruvate formate lyase; FHL, formate hydrogen lyase; 2[H]=NADH/NADPH/FADH2.

Other microorganisms, including *Propionibacterium acidipropionici* (Himmi et al., 2000) and *Anaerobiospirillum succiniproducens* (Lee et al., 2001), ferment glycerol even with no reported propanediol-producing ability, but their metabolic routes for glycerol dissimilation under anaerobic conditions have not been characterized.

5.1.2. Products from glycerol fermentation

The characterization of pathways for glycerol dissimilation in microorganisms that natively ferment glycerol, as well as metabolic engineering and synthetic biology efforts to improve and expand production of native and non-native products in these microorganisms, have enabled the synthesis of valuable fuels and chemicals using glycerol as a feedstock (Clomburg and Gonzalez, 2013; Mattam et al., 2013). Table 5.1 summarizes the portfolio of fuels and chemicals that have been reported to date in native and engineered microorganisms.

Table 5.1. Fuels and chemicals produced from the anaerobic fermentation of glycerol

Products and Organisms	Native/ Engineered	Glycerol Source	Ref.
1,3-Propanediol			
Clostridium butyricum ARK102a	Native	Refined	(Chatzifragkou et al., 2011)
Clostridium acetobutylicum DG1 (pSPD5)	Engineered	Refined	(Gonzalez-Pajuelo et al., 2005)
Escherichia coli	Engineered	Crude	(Tang et al., 2009)
Klebsiella pneumoniae M5aI	Native	Refined	(Jin et al., 2011)
1,2-Propanediol			
Escherichia coli	Engineered	Refined	(Clomburg and Gonzalez, 2011)
Ethanol			
Citrobacter freundii FMCC-207	Native	Crude	(Metsoviti et al., 2012)
Enterobacter aerogenes HU-101	Native	Crude	(Ito et al., 2005)
Escherichia coli	Engineered	Crude	(Yazdani and Gonzalez, 2008)
Klebsiella pneumoniae	Native	Crude	(Oh et al., 2011)
Kluyvera cryocrescens S26	Native	Crude	(Choi et al., 2011)
Succinic acid			
Actinobacillus succinogenes	Native	Crude	(Lee et al., 2001)
Anaerobiospirillum succiniproducens	Native	Crude	(Lee et al., 2004)

Escherichia coli	Engineered	Crude	(Blankschien et al., 2010)
Pasteurellaceae strain DD1	Native	Crude	(Scholten and Daegele, 2008)
Lactic acid			
Escherichia coli	Engineered	Crude	(Mazumdar et al., 2013)
Propionic acid			
Propionibacterium acidipropionici	Native	Refined	(Zhu et al., 2010)
n-Butanol			
Clostridium pasteurianum MBEL_GLY2	Native	Refined	(Moon et al., 2011)

The industrial potential for some of these products is still limited by toxicity, the strict anaerobic conditions needed for fermentation, and the need of supplementation with rich nutrients. Therefore, the use of *in silico* models and systems level tools is vital for continued characterization and improvement of routes leading from glycerol to target products.

Most of the research performed in our lab for glycerol fermentation and the work presented in this chapter are based on using *E. coli* as the quintessential host for industrial production of fuels and chemicals. As shown in Table 5.1, *E. coli* has been engineered in our lab to utilize glycerol as feedstock for the production of 1,2-propanediol(Clomburg and Gonzalez, 2011), ethanol, succinic acid (Blankschien et al., 2010), and lactic acid (Mazumdar et al., 2013; Mazumdar et al., 2010), among others. The work presented below describes efforts in addition to traditional metabolic engineering strategies to better understand and improve fermentative utilization of glycerol in *E. coli.*

5.1.3. Kinetic model for the fermentative metabolism of glycerol in *E. coli*

The development of *in silico* kinetic models can be used to gain a better understanding of the pathways involved in the fermentative metabolism of glycerol and to identify key targets for genetic manipulations that could potentially improve the synthesis of fuels and chemicals. Kinetic models can provide valuable quantitative information about the metabolic network (Simpson et al., 1998) and can set the base for metabolic control analysis (MCA), through which the control structure of the metabolic network can be determined. A kinetic model simulating batch fermentation of glycerol in *E. coli* was recently constructed using mass balances and kinetic expressions for metabolites involved in glycerol fermentative pathways (Cintolesi et al., 2012). MCA performed on this model revealed two key enzymes, glyDH and DHAK, that control the glycolytic flux during glycerol fermentation in *E. coli*. Experimental validation of these results led the implementation of metabolic engineering strategies to increase ethanol production at 20g/L ethanol from crude glycerol, at 92% theoretical maximum (mol/mol).

While, in the past decade, substantial work has been done to understand, characterize and exploit the potential of glycerol as a feedstock for the microbial production of a wide range of chemicals, this work has followed a bottom-up approach, focusing on engineering specific enzymes

and pathways. In this chapter, a system level comparison of the proteome of *E. coli* in different glycerol availability environments is intended to provide a better understanding of glycerol metabolism and identify targets for its improvement and expansion.

5.2. Materials and Methods

Strains, genetic and analytical methods, and procedures for proteomic analysis are detailed in Chapter 3. This section explains culture medium and conditions that are specific to the current chapter.

5.2.1. Culture Medium and Culture Conditions

The minimal medium designed by Neidhardt et al. (1974) with Na_2HPO_4 in

place of K_2HPO_4 and supplemented with 20g/L casamino acids was used as the base

medium. Glycerol addition depended on the culture conditions that were used for comparison: glycerol (10g/L) and no glycerol (0g/L). The medium was added to closed Hungate tubes, and then supplemented with the oxygen indicator resazurin (1 mg/L) (Ferguson and Cummins, 1978) and reducing agent dithiothreitol (DTT, 1mM). Chemicals were obtained from Fisher Scientific (Pittsburg, PA) and Sigma-Aldrich Co. (St. Louis, MO). Closed tube

fermentations were conducted in 17-mL Hungate tubes (Bellco Glass, Inc., Vineland, NJ). Prior to inoculation, oxygen in the medium was removed through the addition of reducing agent (DTT) and stored in an oxygen-free environment (5 % CO_2, 5% H_2, 90% N_2) provided within a BACTRON I Anaerobic Chamber (Sheldon Manufacturing Inc., Cornelius, OR) until the medium was void of color (resazurin remains pink in the presence of oxygen). A single colony was used to inoculate 7 mL of medium in each tube (preculture), which were then incubated at 37 °C with rotation in a LabQuake rotator (Fisher Scientific, Pittsburgh, PA) in an Isotemp Incubator (Fisher Scientific, Pittsburgh, PA) until they reached an OD_{600} of ~0.1. Precultures were transferred back to the anaerobic chamber, in which they were used to inoculate fresh cultures to an initial OD_{600} of 0.01. Cultures were grown for 8-12 hours. When harvesting samples for proteomic analysis, cultures were stopped at 4 hours and tubes corresponding to the same condition were pooled together and harvested as detailed in section 3.3.1.

5.3. Results

While glycerol fermentation pathways and intermediates have been thoroughly studied using a bottom-up approach, omic studies could provide more insights about other impacts that glycerol and its derived metabolites have at a systems level. The following sections describe the proteomic study

of glycerol utilization in a wild-type *E. coli* strain with fully functional pathways for the fermentation of glycerol and in mutant strains with impaired glycerol utilization. A comparison between these strains with and without glycerol availability provided information about cellular processes that became affected by glycerol fermentation and hypothesis as to how glycerol affected the expression of proteins involved in these processes.

5.3.1. Glycerol fermentation in wild-type *E. coli* and mutants with impaired glycerol consumption

In order to study the effect of different glycerol utilization capabilities on protein expression, two *E. coli* strains with fully functional and impaired glycerol utilization pathways were analyzed under anaerobic conditions. *E. coli* BW25113 wild-type (Baba et al., 2006) was used as the control strain and its glycerol consumption capability was compared to that of the alcohol dehydrogenase (AdhE)-deficient strain Δ*adhE.* AdhE was selected as it has been shown to be essential for glycerol fermentation for its involvement in ethanol production, an important route for meeting redox and energy requirements (Murarka et al., 2008). The strains were grown in hungate tubes with MOPS minimal medium with Na_2HPO_4 in place of K_2HPO_4 and supplemented with 20g/L casamino acids. The reducing agent DTT (1mM) and the oxygen-sensitive indicator, resazurin (1mg/L) were also added to the

medium in order to ensure anaerobic conditions. Culture preparation was performed in an anaerobic chamber to facilitate anaerobic conditions. Each strain was studied under two glycerol availability conditions: with (10g/L) and without (0g/L) glycerol, in order to detect changes in protein expression according to changes in glycerol utilization. Complete glycerol absence from the casamino acid mixture was confirmed by 1D proton NMR, in which a sample containing the blank medium (no glycerol) was compared to a glycerol standard, as explained in the Materials and Methods chapter (Chapter 3).

In order to select the experimental conditions that would optimize the amount of valuable information obtained from the proteomic analysis, cell growth and glycerol consumption profiles were constructed for both strains, BW25113 wild-type (WT) and BW25113 Δ*adhE* (Figure 5.2). It is worthy of mention that a Δ*gldA* mutant and a Δ*adhE* Δ*gldA* double mutant were also analyzed, but their glycerol consumption was higher than that of the Δ*adhE* mutant (data not shown). While GldA catalyzes the first step of glycerol dissimilation under fermentative conditions (the conversion of glycerol to dihydroxyacetone) and has been linked to controlling flux during glycerol fermentation (Cintolesi et al., 2012), another metabolic route converting glycerol to glycerol-3-phosphate by means of GlpK is also available and, while slightly impaired, glycerol can still be consumed by the GldA-deficient mutant.

As can be observed in Figure 5.2.a, growth of both strains under both glycerol availability conditions were very similar up to 4 hours of culture, when growth rates of cultures growing in the absence of glycerol began to decrease and they subsequently entered stationary phase. As expected, glycerol-fermenting cultures reached higher ODs due to the availability of the carbon source for higher ATP production. Glycerol consumption for both strains can be seen in Figure 5.2.b. At 4 hours of culture, glycerol consumption for the wild-type strain is only slightly higher than for the Δ*adhE* mutant. However, after 4 hours, the wild-type continues consuming glycerol at a steady rate, while the Δ*adhE* mutant barely consumes any additional glycerol. As has been reported in previous studies (Murarka et al., 2008), this confirms the impairment caused by the absence of *adhE* to anaerobically consume glycerol.

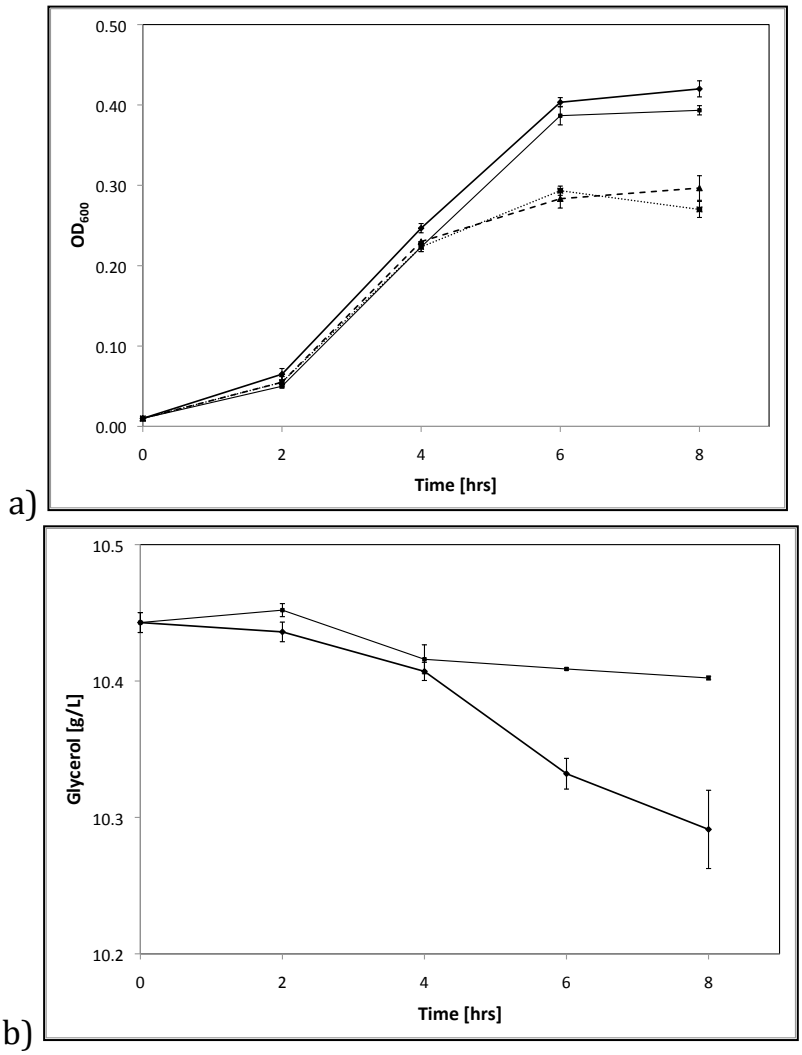


Figure 5.2*. E. coli* (a) cell growth and (b) glycerol consumption profiles. Media was supplemented with casamino acids (20 g/L) and DTT (1mM). 7mL of medium were added to 17mL hungate tubes in anaerobic chamber. [—•—] BW25113WT Gly+; [—▪—] BW25113 Δ*adhE* Gly+; [- ▲ -] BW25113WT Gly-; [···▪···] BW25113 Δ*adhE* Gly-. Gly+, represents glycerol availability conditions (10g/L); Gly-, represents no glycerol availability (0g/L).

The disturbance of glycerol fermentation in the Δ*adhE* mutant can be observed more clearly in Figure 5.3. A 26% and 67.5% decrease in glycerol consumption can be observed for the Δ*adhE* mutant at 4 and 8 hours of culture, respectively.

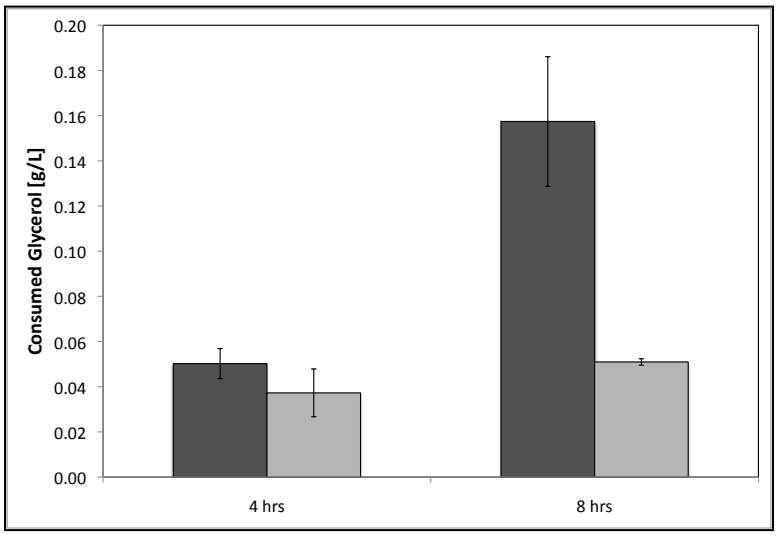


Figure 5.3. Glycerol consumption in different *E. coli* backgrounds. Media was supplemented with casamino acids (20 g/L) and DTT (1mM). 7mL of medium were added to 17mL hungate tubes in anaerobic chamber. Dark bars represent glycerol consumption for BW25113WT, while light bars represent BW25113 Δ*adhE*. A clear impairment of glycerol consumption is observed for the *adhE* deletion mutant.

After comparing the growth characteristics of the four cultures, the 4-hour time-point was selected as the harvest time for samples for proteomic analysis. At this time, all cultures are in mid-exponential phase, in which cells are healthy and at a good protein production stage. Also, the fact that all cultures are at the same stage suggests that the differences in protein expression will be due to differences in glycerol availability and not to different metabolic states of the cells.

5.3.2. Proteomic analysis of *E. coli* under fermentative utilization of glycerol

To study differential expression of proteins during anaerobic glycerol utilization, we performed a proteomic analysis comparing wild-type *E. coli* BW25113 and its Δ*adhE* deletion mutant during mid-exponential growth (OD_{600} around 0.25) in MOPS media supplemented with 20g/L casamino acids in the presence (10g/L) and absence of glycerol as a carbon source, as described in the previous section. Figure 5.4 describes the rationale for each comparison: (1) a comparison between the wild-type strain in the presence (BW25113WT Gly+) and absence (BW25113WT Gly-) of glycerol will provide information about the expression of glycerol utilization pathways at a wild-type level; (2) a comparison between the two strains in the presence of glycerol (BW25113WT Gly+ and BW25113 Δ*adhE* Gly+) will provide information about what other systems become affected when glycerol utilization is impaired; and, finally, (3) a comparison between the two strains in the absence of glycerol (BW25113WT Gly- and BW25113 Δ*adhE* Gly-) will provide a comparison between the two strain backgrounds, independent of glycerol utilization that will be used as a base to filter out differentially expressed proteins arising from differences in genetic background. In addition, comparisons between all conditions with glycerol availability (Gly+)

and all conditions without glycerol availability (Gly-) could also provide information to support the previous comparisons.

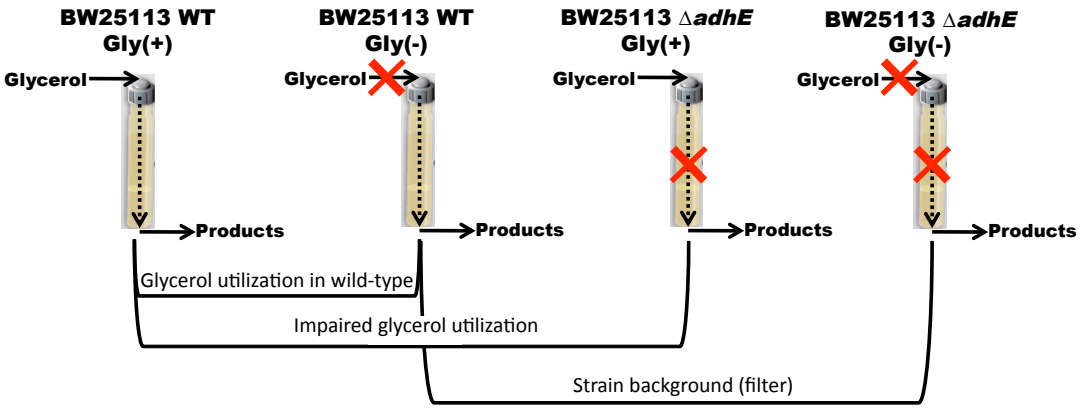


Figure 5.4. Experimental design for comparative proteomic analysis of glycerol fermentation in *E. coli*. Three individual comparisons studied to determine differentially expressed proteins, directly or indirectly, involved in glycerol metabolism. Arrows do not represent any specific reaction. Solid arrows represent glycerol consumption and product synthesis, while dashed arrows represent metabolic pathways leading from substrate to products. Red 'X's represent disruption of glycerol utilization by either not providing glycerol availability (0g/L) or by genetically disrupting its consumption (Δ*adhE*).

Three biological replicates of each condition were analyzed by 2D gel electrophoresis and were compared using Progenesis Same Spots software, as explained in the Materials and Methods chapter (Chapter 3). This software normalized individual spot intensities using the total spot intensity per gel to compensate for variations between gel replicates. The log of each normalized value was then calculated to reduce spot volume to spot deviation dependency and a statistical comparison of the relative spot volumes using ANOVA was performed. In addition of the one-way ANOVA test performed by

Progenesis Same Spots software, a two-way ANOVA test was performed using Matlab in order to take into account differences arising from different strain backgrounds. This analysis provided three p-values corresponding to comparisons by glycerol availability, by strain, and taking into account the interactions between all conditions (Figure 5.4). Spots with p-values lower than 0.05 were considered statistically significant. In order to obtain the spots that were statistically significant only for glycerol fermentation, and not necessarily due to different strain backgrounds, a "filter" was performed in which spots that were statistically significant in the comparison of BW25113 WT Gly- versus BW25113Δ*adhE* Gly- were eliminated from the analysis. Following this analysis, out of 897 total protein spots, 78 spots had a p-value $\leq$ 0.05 between the comparisons of interest. The spots that were abundant at such a level as to be detected in a coommassie blue-stained gel and that were differentially expressed with a p-value $\leq$ 0.05 were excised and analyzed by MS (22 spots).

Table 5.2. Differentially expressed proteins during glycerol fermentation.
Proteins were identified by Mass Spectrometry and gel matching to online databases. Red and green boxes represent downregulated and upregulated proteins during glycerol fermentation, respectively. White boxes show no change in expression.

Protein	p-value	Fold Change	
		BW25113WT Gly+/ BW25113WT Gly-	BW25113WT Gly+/ BW25113ΔadhE Gly+
Hypothetical protein	0.0099	-3.8	X
DHAM	0.0144	-2.4	1.1
PEPQ	0.0402	1.4	2.7
PURE	0.0215	1.0	1.1
UCPA	0.0138	1.1	-1.5
FOLP	0.0113	1.0	-1.5
GLOA	0.0250	-1.5	-1.5
MTN	0.0212	1.0	-1.5
TALB	0.0251	1.0	-1.4
GLPB	0.0144	-2.4	1.1
GLYA	0.0426	1.7	2.0
AROC	0.0024	1.7	1.6
ACEA	0.0354	-2.0	1.0
CARA	0.0429	1.6	1.2
RPSE	0.0158	-1.4	-1.6
PHOP	0.0324	-1.2	-1.5
ENO	0.0139	-1.3	1.0
NUSA	0.0143	-2.1	-1.1
FABH	0.0396	-1.1	-1.5
DAPD	0.0162	-1.3	-1.5
EFP	0.0223	-1.3	-1.2
DEOD	0.0106	1.0	-1.7
GLYA	0.0428	1.5	-1.1
OMPF	0.0253	1.5	1.3
POXB	0.0214	1.3	7.0
PHOE	0.0018	1.8	1.2
TPX	0.0036	1.9	1.1

These 22 spots were subjected to MALDI-TOF/MS analysis (Figure 5.6), followed by homology search using MASCOT and identification from the NCBI database. Five additional spots with differential abundance were identified by matching to *E. coli* master gels from the ExPASy online database (Gasteiger et al., 2003; Wilkins et al., 1999). The 27 proteins that were identified as

differentially expressed during fermentative utilization of glycerol have different roles in cellular metabolism, structure, and regulation. A more detailed examination of the roles of these proteins on glycerol fermentation in *E. coli* is presented below.

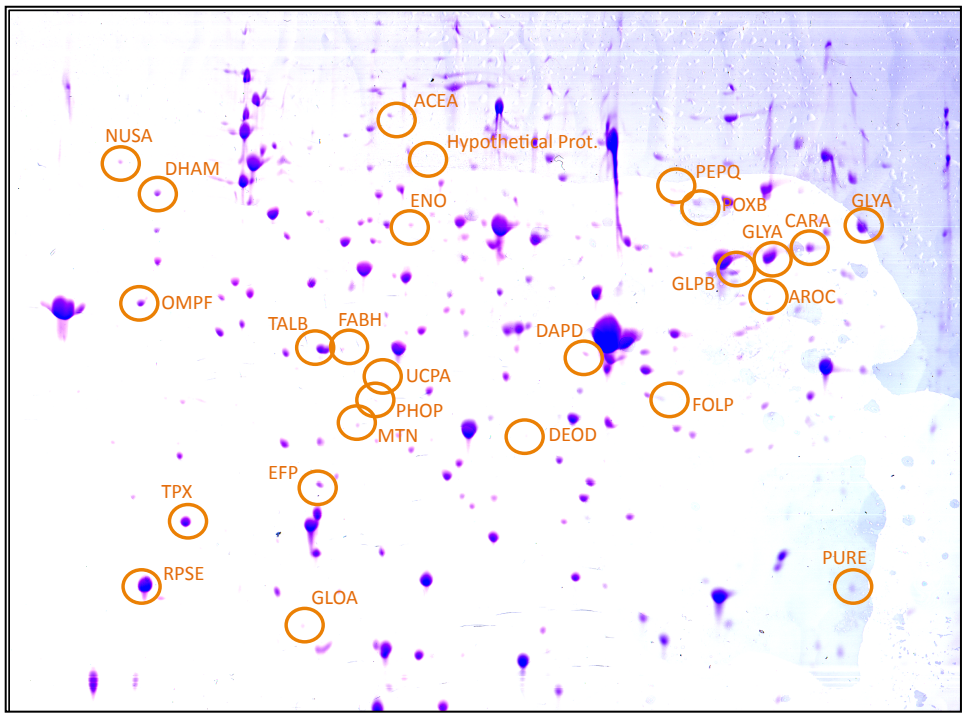


Figure 5.5. Differentially expressed proteins during glycerol fermentation in *E. coli*. Coommassie blue-stained 2D gel showing protein spots that were excised and analyzed by mass spectrometry analysis.

5.3.3. Proposed roles of differentially expressed proteins during fermentative utilization of glycerol

Cellular functions of the 27 differentially expressed proteins have been investigated and several hypotheses have arisen from information found in the literature. Proteins that were differentially expressed during glycerol

fermentation had functions related to glycerol and central metabolism, nucleotide and amino acid biosynthesis and degradation, transport and structure, oxidation reduction processes, and regulation. A general overview of cellular functions for these proteins is presented in Table 5.3 and 5.4, while more detailed descriptions and hypotheses are presented in the sections below.

Table 5.3. Biological roles of differentially expressed proteins during glycerol fermentation in *E. coli*.

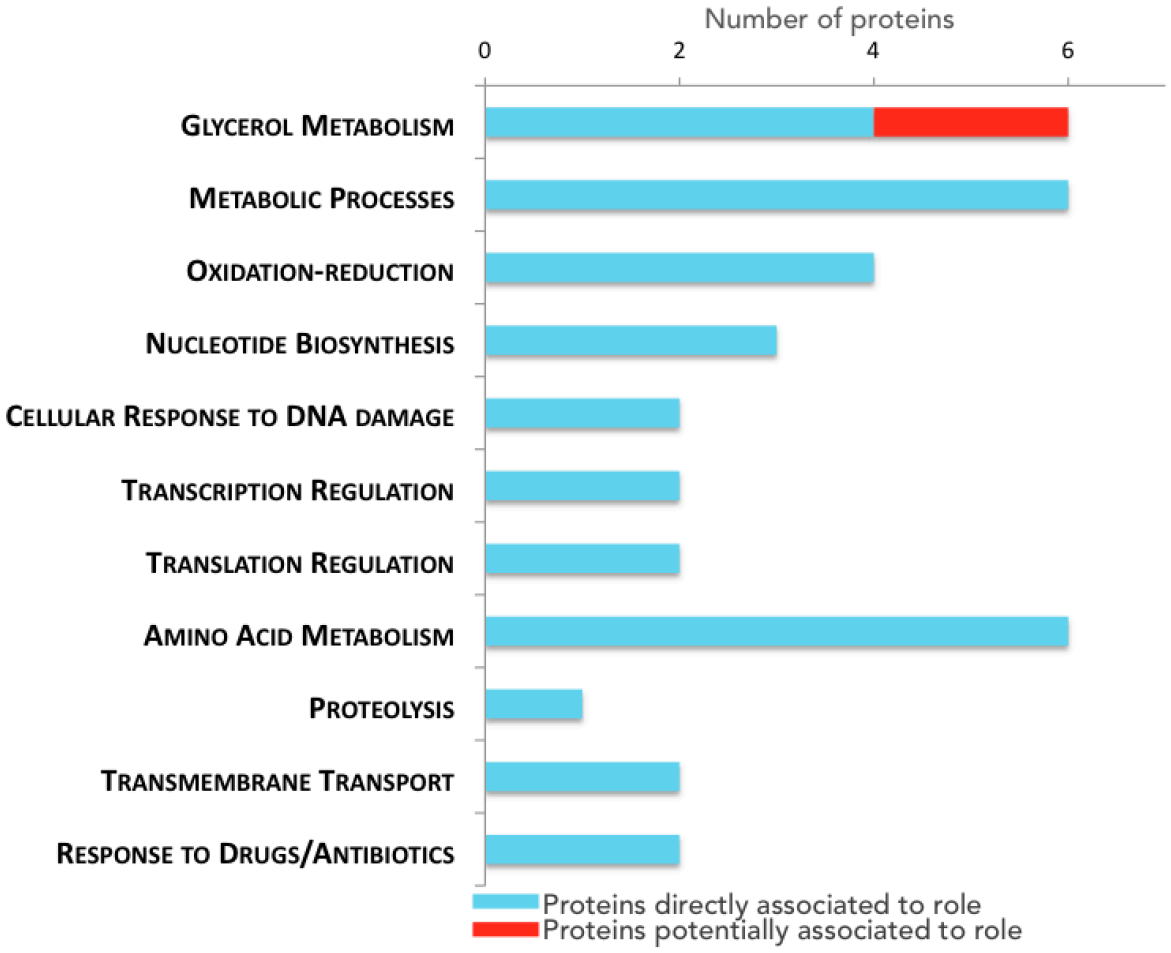
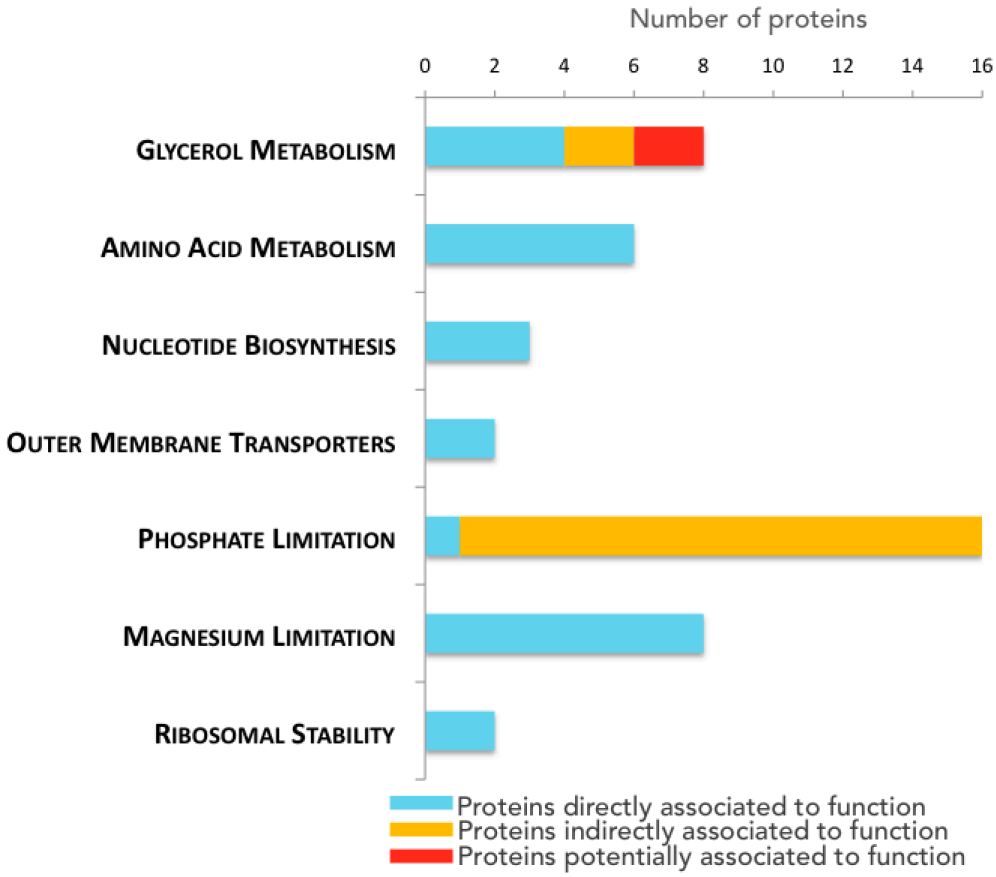


Table 5.4. Cellular systems affected during glycerol fermentation



5.3.3.1. Enzymes involved in glycerol metabolism

As would be expected when comparing glycerol fermenting to non-glycerol fermenting cultures, some of the differentially expressed proteins arising from this analysis are components of glycerol dissimilation pathways in *E. coli.* In this study, three distinctive proteins involved in glycerol utilization were upregulated in glycerol fermenting cultures. Two of those, subunit M of the dihydroxyacetone kinase complex (DhaM) and subunit B of

the glycerol-3-phosphate (G3P) dehydrogenase complex, are responsible for key reactions that channel glycerol to glycolytic intermediates (Figure 5.6).

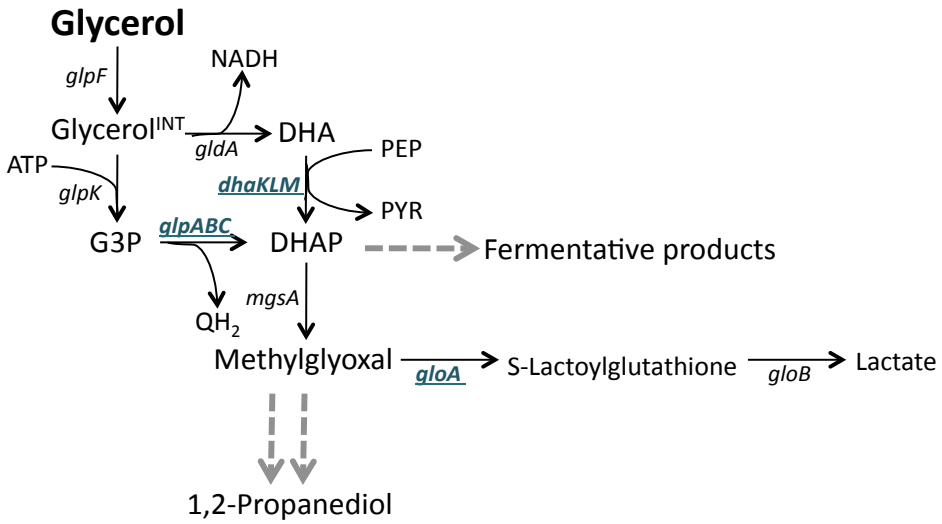


Figure 5.6. Pathways involved in glycerol transport and utilization under anaerobic conditions. Broken lines indicate multiple reactions. Reactions are represented by the genes encoding the enzymes. Enzymes in blue were differentially expressed during glycerol fermentation. Abbreviations: INT, intracellular; G3P, glycerol-3-phosphate; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate.

The dihydroxyacetone kinase complex encoded by *dhaKLM* catalyzes the conversion of dihydroxyacetone (DHA) to dihydroxyacetone phosphate (DHAP), following glycerol dehydrogenation by glyDH-I (Gonzalez et al., 2008). DhaK has been identified as a key enzyme for anaerobic utilization of glycerol in several reports from our group, covering *in vivo* analyses and *in silico* metabolic control analysis (MCA) (Cintolesi et al., 2012; Gonzalez et al., 2008; Yazdani and Gonzalez, 2008). The M subunit of this complex is a membrane bound protein that serves as the phosphoryl donor of the DHAK

reaction (Paulsen et al., 2000). It consists of three phosphotransferase system (PTS) domains: the N terminus, similar to the mannose transporter in PTS (IIA), the middle domain, similar to HPr; and the C terminus, which is similar to enzyme I (EI) (Deutscher et al., 2006). These domains are phosphorylated by phosphoenolpyruvate (PEP) in EI- and HPr-dependent reactions, from which the phosphoryl group is transferred to a DhaL-ADP complex, and ultimately to a DhaK-DHA complex in a total of seven steps (Deutscher et al., 2006; Gutknecht et al., 2001). The DHAKLM complex is regulated by DhaR, which is activated in the presence of DHA. DhaM expression was upregulated in the wild-type cultures fermenting glycerol with respect to the wild-type cultures grown without glycerol, agreeing with previous studies from our group that reported its crucial role for fermentative utilization of glycerol.

The G3P dehydrogenase complex encoded by *glpABC* has been linked to a glycerol dissimilation pathway that is active under anaerobic conditions (Cole et al., 1988; Durnin et al., 2009; Lin, 1976). It catalyzes the conversion of G3P to DHAP, after glycerol has been activated by the kinase GlpK. In this study, GlpB showed upregulated expression in wild-type cultures in the presence of glycerol with respect to the same strain without glycerol. While several studies have reported the glyDH-DHAK route as being the key route for glycerol dissimilation under anaerobic conditions (Cintolesi et al., 2012; Clomburg and Gonzalez, 2011; Gonzalez et al., 2008), our experiments show

that the GlpK-GlpABC route is still being expressed during glycerol fermentation, even though it may not play a key role in the metabolic pathway. In fact, *in silico* metabolic flux analysis (MFA) performed in our lab showed flux going through both pathways (Clomburg, 2012) (Table 5.5). These findings led to test enzyme activities, which were found to be at similar levels for the two pathways, indicating that they were both active during glycerol fermentation (Clomburg, 2012).

Table 5.5. Measured activities of enzymes involved in glycerol dissimilation. (Clomburg, 2012)

Enzyme Assayed (*Gene*)	Activity [a,b] (µmol/mg protein/min)
glycerol dehydrogenase (*gldA*)	0.132 ± 0.008
PEP-dependent dihydroxyacetone kinase (*dhaKLM*)	0.051 ± 0.002
glycerol kinase (*glpK*)	0.102 ± 0.008
anaerobic glycerol-3-phosphate dehydrogenase (*glpABC*)	0.120 ± 0.007
aerobic glycerol-3-phosphate dehydrogenase (*glpD*)	ND[c]

[a]Values reported as average ± standard deviation of triplicate assays
[b]Activities measured in wild-type BW25113 after 72 hours growth
[c]ND: Not detectable (minimum detectable activity was 0.001µmol/mg protein/min)

In addition, the inability to completely stop glycerol consumption in a Δ*gldA* mutant, suggests an alternative route for glycerol dissimilation is still active in the cells (see section 5.3.1).

On the other hand, expression of DhaM and GlpB was not different when comparing the wild-type strain and the Δ*adhE* mutant, both in the presence of glycerol. While glycerol metabolism is impaired in the Δ*adhE* mutant, data showed that there was still a small amount of glycerol being

fermented. These results confirm that both, DhaM and GlpB, are expressed at wild-type levels in the deletion mutant.

Following the dissimilation of glycerol, dihydroxyacetone phosphate is then converted to methylglyoxal to enter the reductive branch of glycerol fermentation that leads to 1,2-PDO synthesis, or it can continue glycolytic flux towards pyruvate and other fermentation products. Methylglyoxal is a toxic metabolic intermediate whose accumulation, even at low levels, can lead to growth inhibition and cell death (Booth et al., 2003). The glyoxalase system, composed of glyoxalase I (Glx-I) and glyoxalase-II (Glx-II) has been identified as the main methylglyoxal detoxification pathway in *E. coli* (MacLean et al., 1998; Rose and Nowick, 2002). Results from our proteomic analysis showed upregulation of Glx-I (*gloA*) in the wild-type culture under glycerol fermentation with respect to both conditions, wild-type with no glycerol and Δ*adhE* mutant with glycerol. The comparison of the wild-type strain under glycerol and no-glycerol conditions agrees with the established glycerol fermentation model, in which methylglyoxal produced from DHAP is converted to lactate using the glyoxalase complex as the main detoxification route (Clomburg and Gonzalez, 2011; Gonzalez et al., 2008). On the other hand, although some enzymes catalyzing the first steps of glycerol dissimilation were expressed at wild-type levels in the Δ*adhE* mutant, glycerol fermentation was still impaired and methylglyoxal concentrations

may not have reached sufficient levels to trigger the expression of the detoxifying glyoxalase complex. This could indicate a preferential route for DHAP towards PEP at low fermentation levels.

Besides these three essential proteins involved in glycerol fermentation, an additional differentially expressed protein could also be potentially involved in glycerol metabolism in *E. coli.* The protein encoded by *ucpA*, a predicted dehydrogenase (Reed et al., 2003) and NAD(P)-binding oxidoreductase (Serres et al., 2001), was downregulated in the Δ*adhE* mutant with respect to the wild-type, both in the presence of glcyerol. Although at a lower level, this protein was also downregulated in the wild-type under glycerol-fermenting conditions when compared to the same phenotype with no glycerol. Even though not much is known about *ucpA*, homologues of the protein have been reported to contain a proposed NAD-binding site with homology to short-chain alcohol dehydrogenases (Sirko et al., 1997). Effects of *ucpA* on furan tolerance have also been studied, showing that *ucpA* overexpression conferred an increased tolerance to furan (Wang et al., 2012). In this latter study, a range of substrates including glycerol, ethanol, and acetaldehyde, among others, were tested for *ucpA* activity with no increased activity observed for any of the substrates, but these tests were performed on whole-cell lysates, in which enzymatic activities for *ucpA* could have been masked by other oxidoreductases, depending on the conversion rate of *ucpA*.

The redox constraints imposed by anaerobic metabolism of glycerol make this a very interesting finding and the fact that differential expression of this predicted oxidoreductase is more evident in the *adhE* deletion mutant, in which *gloA* was also downregulated, suggests that it could be involved in one of the reactions branching out from methylglyoxal. As explained above, downregulation of Glx-I (*gloA*) and now *ucpA,* while DhaM and GlpB are upregulated suggest diversion of flux in the DHAP node away from methylglyoxal. UcpA could potentially be an oxidoreductase involved in the conversion of methylglyoxal to 1,2-PDO. Enzyme activity assays with the purified enzyme should be performed in order to test for dehydrogenase activity using intermediates from glycerol oxidation to DHAP and 1,2-PDO synthesis as substrates.

While not directly involved in glycerol dissimilation pathways, Tpx is a lipid hydroperoxide peroxidase which acts as the principal lipid antioxidant under anaerobic conditions (Cha and Kim, 1999; Cha et al., 2004). Glycerol fermentation in *E. coli* occurs in a high redox environment, posing additional metabolic constraints to the cells (Durnin et al., 2009; Gonzalez et al., 2008), and providing the environment for changes in peroxidase expression. Another constraint that has been previously presented is that fermentative metabolism of glycerol leads to limitations in PEP, due to coupling of DhaKLM with the conversion of PEP to PYR (Gonzalez et al., 2008). Upregulation of

DhaM during glycerol fermentation in the wild-type and the Δ*adhE* mutant, which showed impaired glycerol utilization, confirms that the DhaKLM complex plays a key role in glycerol dissimilation and supports reports about PEP limitation. Enolase (Eno), which catalyzes the conversion of 2-phosphoglycerate to PEP (Halliday et al., 2010; Spring and Wold, 1971), was also upregulated in glycerol fermenting conditions, suggesting a connection between the two enzymes in order to meet PEP requirements.

5.3.3.2. Outer membrane transporters

The outer membrane in *E. coli* is a protective barrier that is composed of lipopolysaccharides, phospholipids, and proteins (Lugtenberg, 1981; Molloy et al., 2000). Many outer membrane proteins, called porins, serve as pores or channels that facilitate transport of nutrients across the membrane. While some of these channels allow passage of a wide variety of molecules, others are selective towards molecules with a specific structure or charge. In addition, expression of many of these porins is regulated by a response to changes in intracellular or extracellular environments (Fajardo et al., 1998; Liu and Ferenci, 1998; Meyer et al., 1990). In this study, outer membrane porins F (OmpF) and E (PhoE) were two of the differentially expressed proteins during glycerol fermentation. Both proteins were downregulated in the wild-type strain in the presence of glycerol, with respect to the wild-type

in the absence of glycerol and the Δ*adhE* mutant in the presence of glycerol. Although both, OmpF and PhoE, are subject to osmotic regulation (Forst et al., 1989; Meyer et al., 1990), the negligible osmotic stress presented by glycerol may not be sufficiently strong to trigger such a response (Alemoham.Mm and Knowles, 1974).

Outer membrane porins C and F (OmpC and OmpF) are two of the main non-specific outer membrane channels, which facilitate the permeation of mostly hydrophilic solutes (Hoenger et al., 1993; Nakae, 1976). The total expression of these two porins remains constant during osmotic regulation, with OmpF being downregulated and OmpC being upregulated in high-osmolarity conditions, and vice versa in low-osmolarity (Yoshida et al., 2006). Besides responding to osmotic stress, OmpF has also been associated with voltage-gating and acid stress responses (Muller and Engel, 1999; Robertson and Tieleman, 2002). A study by Chagneau, *et al.* on the regulation of pH-sensitive genes in the maltose and porin regulons suggested an effect of *glpFKX* (encoding the glycerol diffusion facilitator (GlpF), glycerol kinase (GlpK), and a fructose 1,6-biphosphatase (GlpX)) expression on the *ompF* gene (Chagneau et al., 2001). In this study, a mutation leading to a decrease in *glpFKX* expression resulted in an increase in *ompF*, *malt*, and MalT-dependent gene expression during growth in acidic medium. Conversely, *glpFKX* amplification lowered *ompF* transcription by two-fold, while *ompC* expression

was not significantly modified. These results confirmed that at least one of the proteins encoded by the *glpFKX* operon and expressed at the wild-type level could contribute, directly or indirectly, to the repression of *mal* and *ompF*. All three of these proteins have been shown to be active and important for glycerol fermentation (Clomburg, 2012; Donahue et al., 2000; Lin, 1976). While the mechanism or the reason for such an effect are unknown, one hypothesis could be that when glycerol is present as a substrate and *glpFKX* is expressed, *ompF* is repressed in response to nutritional needs for glycerol metabolism. For example, OmpF has been shown to possess different channel specificities, allowing transport of small, highly charged ions (such as Mg^{2+}, Ca^{2+}), and large ions (such as Cs^{+}) (Miedema et al., 2006; Queralt Martin et al., 2013). In future sections, the differential expression of proteins that require Mg^{2+} as a cofactor is presented and may suggest an unbalance of Mg^{2+} levels in the cells during glycerol fermentation. If this is true, differences in OmpF expression could be responding to such fluctuations in an attempt to maintain ion balances inside the cells. Analysis of the nutrients in the medium could be performed in wild-type and *ompF* mutant strains (deletion and overexpression) to determine if there are any changes in medium composition when the expression of this porin is changed.

On the other hand, PhoE, also named OmpE, is an outer membrane porin in *E. coli* which facilitates efficient diffusion of phosphate and

phosphate-containing compounds across the outer membrane and is induced by phosphate limitation (Korteland et al., 1982). Previous studies in our group have demonstrated that a low phosphate culture medium is key for the anaerobic fermentation of glycerol (Gonzalez et al., 2008). Downregulation of PhoE during glycerol fermentation may be a response of the cells to prevent entrance of phosphate compounds in order to avoid their detrimental effect on glycerol metabolism. In order to confirm this hypothesis, PhoE expression in glycerol fermenting conditions and in a glycerol-free medium with a higher-phosphate concentration can be further compared by SDS-PAGE.

5.3.3.3. 1-Carbon metabolism: Activated methyl cycle (AMC) and methionine metabolism

Methylation of essential biological molecules, such as nucleic acids, lipids, and proteins, is of crucial importance for many biochemical processes for the development and function of organisms. The activated methyl cycle (AMC) is a central metabolic pathway responsible for methylation of cellular components and the recycling of sulfur-containing amino acids (Figure 5.7) (Chiang et al., 1996; Halliday et al., 2010). Several proteins that were differentially expressed during glycerol fermentation in this study are, directly or indirectly, involved in the AMC and in pathways leading to methionine biosynthesis (Mtn, FolP, GlyA, AroC). While protein Mtn directly

catalyzes one of the reactions of the AMC leading to S-ribosylhomocysteine (Cornell et al., 1996), FolP, GlyA, and AroC are involved in the synthesis of intermediates leading to folate and tetrahydrofolate production, which are needed for methionine synthesis (Salem and Foster, 1972). These proteins are shown in Figure 5.7, next to the pathways surrounding the AMC in which they are involved.

In *E. coli* and other bacteria, the AMC also serves as a metabolic pool critical for the formation of quorum-sensing signaling molecules (Vendeville et al., 2005). Quorum-sensing is a cell-to-cell signaling process that allows *E. coli* to collectively control gene expression and synchronize activities that benefit the population. This process is achieved through the production, secretion, and detection of quorum-sensing signaling molecules, also known as autoinducers (Xavier and Bassler, 2005). Autoinducer-2 (AI-2) is derived from S-ribosyl-L-homocystein (SRH), an intermediate from the AMC, which is a product of the reaction catalyzed by Mtn. A recent screening analysis to detect mutants unable to internalize AI-2, revealed *lsr* and *glpD* mutants to have this effect (Xavier and Bassler, 2005). Further studies of these mutants led to the conclusion that G3P, and to a lesser extent, DHAP and glycerol repress expression of the *lsr* operon, which is responsible for internalization and phosphorylation of AI-2. As was explained in section 5.3.3.1, it is possible that impaired glycerol metabolism observed in the Δ*adhE* mutant is causing

accumulation of G3P and DHAP, and increasing pools of these metabolites could be in turn, repressing *lsr* expression and causing accumulation of AI-2 in the medium.

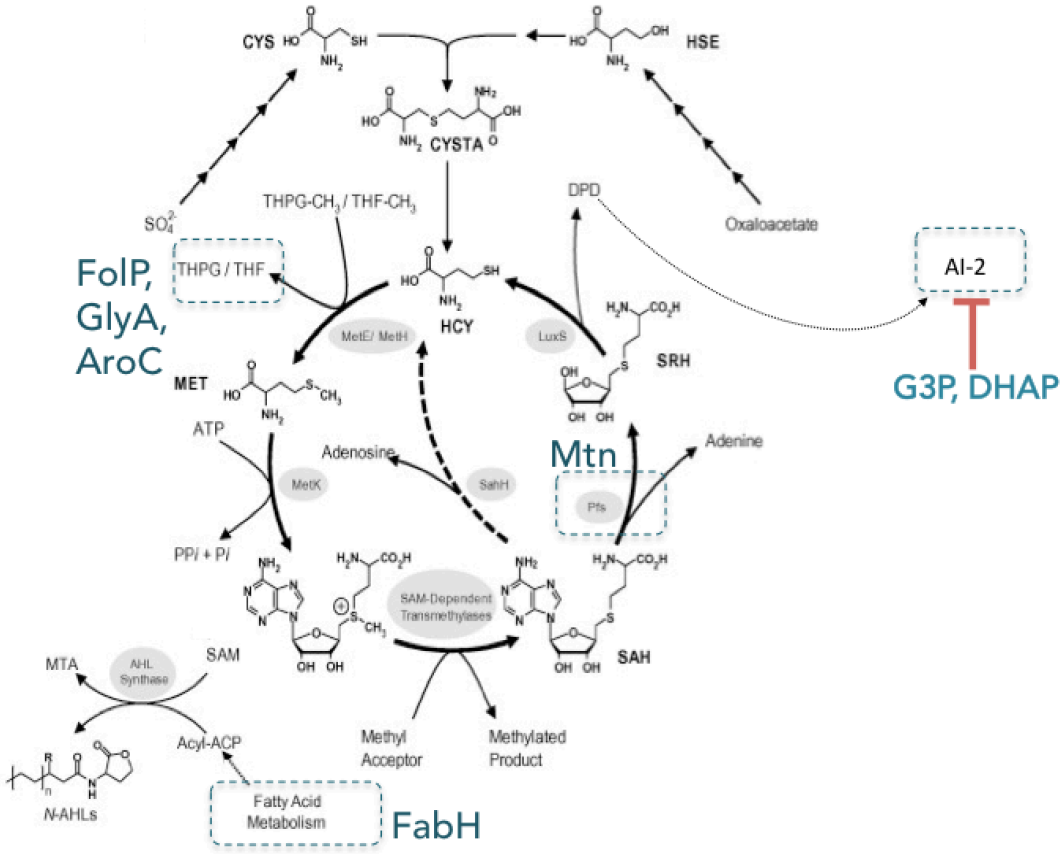


Figure 5.7. Overview of activated methyl cycle (AMC). Diagram of the AMC indicating the pathways responsible for recycling and replenishment of this key metabolite pool. Enzymes, in shaded ovals, are shown adjacent to their relevant pathway. The diagram shows the role of the AMC in the biosynthesis of N-acylhomoserine lactone (N-AHL)- and autoinducer-2 (AI-2)-type quorum sensing signaling molecules. Proteins that were differentially expressed under glycerol fermentation and that are related to the AMC are shown in blue next to the pathways in which they are involved, shown in dashed-lined boxes.
Abbreviations: THPG-CH_3, N^5-methyltetrahydropteroyltriglutamate; THPG, tetrahydropteroyltriglutamate; THF-CH_3, N^5-methyltetrahydrofolate; THF, tetrahydrofolate; ATP, adenosine triphosphate; PP_i + P_i, diphosphate + phosphate; SO_4^{2-}, sulfate; DPD, 4,5-dihydroxyl-2,3-pentanedione; Acyl–ACP, acyl–acyl carrier protein; MTA, methylthioadenosine. For N-AHLs, *n* (length of acyl chain) = 2, 4, 6, 8..; R(functional group) = –OH , O, –H. (Halliday et al., 2010)

Another study reported that LuxS (*luxS*), the enzyme that directly mediates the production of AI-2, and Mtn (*pfs*) are both required for AI-2 production (Beeston and Surette, 2002). Mtn also catalyzes the production of 5'-methylthioribose (MTR) and adenine from 5'-methylthioadenosine (MTA), a potent inhibitor of polyamine synthetases and S-adenosylmethionine (SAM)-requiring reactions (Della Ragione et al., 1985). Regulation of *luxS* expression has been studied in more detail and has been proven to not occur at the transcription level (Xavier and Bassler, 2005). Instead, both, *luxS* and *pfs,* are proposed to be regulated by substrate availability. Results from this proteomic analysis may suggest repression of *pfs* by accumulation of one of its products, SRH, due to repression of LuxS activity by accumulation of AI-2. If Mtn repression does occur in this way, accumulation of its other substrate, MTA could be disrupting other reactions involved in methylation pathways. In order to investigate this hypothesis, two products can be quantified to gain insights on the activity of Mtn, MTR, which is its direct product in the degradation of MTA and is excreted to the medium, and AI-2, the autoinducer which derives from the AMC. Further analysis could include investigation of deletion mutants corresponding to key steps in the AMC. Quantifying accumulation of G3P or DHAP could also provide valuable insights on the ramification of metabolism in strains with impaired glycerol fermentation.

Another type of quorum-sensing molecules made by Gram-negative bacteria, N-acylhomoserine lactones (AHLs), are generated from the AMC through SAM and various intermediates from fatty acid metabolism (Hoang et al., 2002). One of these intermediates is acyl-ACP, which is involved in the activation steps of fatty acid biosynthesis and directly affects FabH expression. Differential expression of FabH in this study was surprising at first, but the described changes in the AMC during glycerol fermentation could be responsible for this observation.

Amino acid biosynthetic pathways are key to conserve essential metabolite pools for cellular function. In addition to observing differential expression of proteins involved in the AMC, proteins FolP, GlyA, AroC, and PepQ were also affected and can be linked to different reactions surrounding methionine biosynthesis and degradation. FolP catalyzes the formation of dihydropteroate, which is subsequently converted to tetrahydrofolate, an essential intermediate in the synthesis of methionine and other amino acids (Talarico et al., 1991). On the other hand, GlyA catalyzes the conversion of serine to glycine, transferring a methyl group to tetrahydrofolate, while forming 5,10-methylene-tetrahydrofolate (5,10-mTHF), which is the major source of C1 units in the cell (Schirch et al., 1985). Therefore GlyA is a major enzyme in the biosynthesis of methionine, among other important molecules in the cell. In addition, protein AroC is involved in chorismate biosynthesis,

which leads to the biosynthesis of aromatic amino acids (Charles et al., 1990). Synthesis of aromatic amino acids has high PEP requirements and results in net production of reducing equivalents from glycerol, both of which are affected during glycerol metabolism due to limitations in PEP and a high redox environment (Gonzalez et al., 2008). It is also important to note that some bacteria, including *E. coli*, possess a *p*-aminobenzoate synthase multi-enzyme complex through which they can convert chorismate to *p*-aminobenzoate and pyruvate (Green et al., 1992). *p*-Aminobenzoate is then converted to tetrahydrofolate, which as stated above, is important for methionine biosynthesis. Alterations in amino acid biosynthetic pathways can also be reflected in the differential expression of PepQ, a proline dipeptidase, which hydrolyzes dipeptide substrates containing a proline residue at the carboxy-terminal (Park et al., 2004). Due to the results explained above, analyzing the composition of amino acids in the media could provide valuable information about the pathways that are being affected by glycerol fermentation, but the fact that the media we used in this study is supplemented with casamino acids makes it somewhat difficult to tie production or consumption of an amino acid solely to a specific pathway.

It is important to mention that simultaneous biosynthesis and degradation of amino acids and nucleotides was reported in a genome-scale model simulating glycerol fermentation (Cintolesi et al., 2014). In their study,

Cintolesi, *et al.* reported flux through several futile, energetically expensive pathways that were acting as electron sinks to achieve redox balance. Since activity of these simultaneous pathways had not been reported for wild-type *E. coli,* the model was curated to eliminate the reactions involved in these pathways. These reactions included production of spermidine and MTR (catalyzed by Mtn), nucleotide degradation, and amino acid degradation and export, among others. In addition, to Mtn, involved in methionine degradation, our proteomic analysis revealed differential expression of PurE, DeoD, and CarA, involved in nucleotide biosynthesis and degradation pathways (Jensen and Nygaard, 1975; Meyer et al., 1992; Piette et al., 1984). Differential expression of these proteins during glycerol fermentation may reveal that, contrary to what has been thought, the cells are actually activating these simultaneous pathways to achieve redox balance or other vital functions.

5.3.3.4. Growth under phosphate-limiting conditions

Phosphate is an essential nutrient that plays a key role in numerous biological processes. It is a component of many biological molecules, a component of membrane lipids, and occupies a central role in cellular energy metabolism. Therefore, phosphate assimilation and metabolism pathways are of critical importance for cell survival. Phosphate regulation is also a very important cellular process, since a great number of transcriptional regulators

use phosphate as an activator (Shinagawa et al., 1983). PhoB is the transcriptional activator for the genes in the *pho* regulon as well as for *phoB* itself and responds to phosphate level in the medium (Yamada et al., 1989). Phosphate limitation has proven to be crucial for glycerol fermentation in *E. coli* (Gonzalez et al., 2008) and the phosphate-limiting medium used in this study could be responsible for the differential expression of several proteins during glycerol fermentation. In addition to thirteen proteins, directly or indirectly, regulated by PhoB, several phosphate-binding transcriptional regulators have come up during research of differentially expressed proteins (Figure 5.8).

Signal	Two-Component System	Proteins Regulated by Two-Component System
Phosphate Control	PhoB-PhoR	**OmpE (PurE, GlyA, GlpB, PoxB, AceA, CarA, RpsE, Eno, NusA, DapD, FabH, DeoD)**
Osmolarity/pH	OmpR-EnvZ	**OmpF**
Magnesium Levels	**PhoP**-PhoQ	
Catabolite Regulation	CreB-CreC	**TalB**

Figure 5.8. Differentially expressed proteins, directly or indirectly, involved in two-component regulatory systems. Proteins in bold were differentially expressed in our study. Underlined proteins represent the regulator in the two-component system. Proteins in parenthesis represent proteins that are indirectly regulated by two-component systems (i.e. the two-component system regulates other genes in the gene regulation cascade for these proteins).

Two-component systems serve as stimulus-response coupling mechanisms to allow *E. coli* to sense and respond to environmental

conditions (Stock et al., 2000). These systems consist of a histidine protein kinase (HK), and a response regulator protein (RR). In most cases, the activity of the HK is controlled by extracellular stimuli, which control the rate of phosphorylation. Once phosphorylated, the HK transfers the phosphoryl group to a cognate RR, which then mediates the output of the signaling pathway (Amin et al., 2014) (Figure 5.9). The chemistry of two-component systems involves three phosphotransfer reactions and two phosphoprotein intermediates:

1. Autophosphorylation of the sensor kinase
2. Phosphotransfer from sensor kinase to response regulator
3. Dephosphorylation of the response regulator

All three reactions require divalent metal ions, with Mg^{2+} presumably being the most relevant ion (Stock et al., 2000).

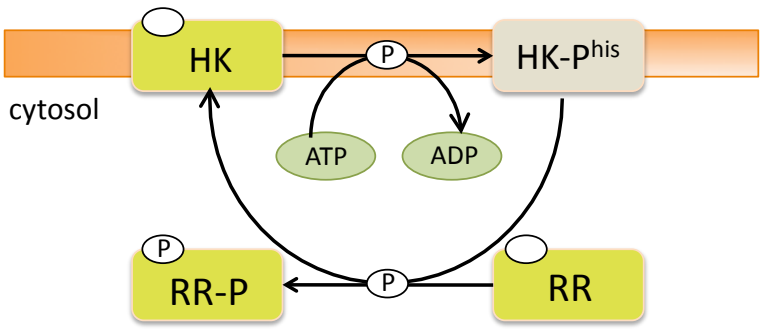


Figure 5.9. Phosphotransfer reactions of two-component regulatory systems. Abbreviations: HK, histidine protein kinase; HK-P^{his}, phosphorylated HK; RR, response regulator; RR-P, phosphorylated RR.

Although these regulators are activated by different signals and a direct link is unclear, differences in Mg^{2+} availability (discussed in the following section) during glycerol fermentation could be affecting two-component regulatory systems. Since these systems are key for genetic regulation, the relation between glycerol fermenting conditions and expression and functionality of two-component regulatory systems should be further explored.

5.3.3.5. Activation of glycerol fermentation pathways could cause magnesium-limiting conditions

Magnesium ion (Mg^{2+}) is an essential nutrient in cellular systems, since it acts as a cofactor for many enzymes and is key in a great number of metabolic reactions involving ATP (Reinhart, 1988). Seven of the differentially expressed proteins under glycerol fermentation use Mg^{2+} as a cofactor for enzymatic activity or structural stability. Five of these proteins were upregulated during glycerol fermentation (AceA, Eno, Efp, FolP, and DapD), while two other proteins were downregulated under the same conditions (PoxB and CarA). In addition, PhoP, a member of the PhoP-PhoQ two-component regulatory system, which is involved in *E. coli* adaptation to low Mg^{2+} concentrations (Groisman, 2001; Yamamoto et al., 2002) was upregulated during glycerol fermentation. This regulatory system consists of

the sensor PhoQ, which detects extracellular Mg^{2+} level and the response regulator, PhoP, which becomes phosphorylated by PhoQ under low Mg^{2+} levels (Groisman, 2001). These results imply that expression of proteins, directly or indirectly, involved in glycerol fermentation could be causing limited levels of Mg^{2+} for cellular function.

In addition, Mg^{2+} is also known to be essential for ribosomal stability (Gestelan.Rf, 1966; Goldberg, 1966). Low Mg^{2+} levels have been shown to cause unfolding of the ribosomes in *E. coli* (Gestelan.Rf, 1966), while the S5 subunit is one the subunits which have been reported to be important for ribosome structure and translational fidelity (Lodmell and Dahlberg, 1997). Moreover, protein EF-P was also upregulated during glycerol fermentation in this study. EF-P is an elongation factor that stimulates the formation of peptide bonds by alleviating ribosome stalling at polyproline stretches (Doerfel et al., 2013). It has been reported to be sensitive to Mg^{2+} concentrations and to require both 30S and 50S subunits for activity(Glick and Ganoza, 1976). Upregulation of the 30S ribosomal subunit S5 (RpsE) and the elongation factor EF-P in glycerol fermenting cultures further supports the hypothesis of limited magnesium availability during anaerobic glycerol utilization.

Mg^{2+} limitation during glycerol fermentation can be tested by analyzing a Δ*phoP* mutant under the fermentative conditions used in this study. Such a

mutant has been shown to have completely impaired growth in studies with *Salmonella typhimurium* (Vescovi et al., 1996). In addition, restoring growth by Mg^{2+} media supplementation would confirm this hypothesis. Mg^{2+}-dependency of glycerol fermentation can be further investigated by analyzing cell growth and glycerol utilization in media supplemented with different concentrations of Mg^{2+}. Media formulation proved to be key initially in achieving glycerol fermentation in *E. coli* (Gonzalez et al., 2008) and Mg^{2+} supplementation could result in further improvement of glycerol utilization rates.

5.4. Conclusions

Glycerol has gained increased attention as a substrate for biorefineries in recent years, due to an increase in the production of biodiesel and bioethanol, both of which co-produce glycerol as a by-product. Its high degree of reduction also makes it a valuable carbon source for the production of reduced chemicals. Recent studies of glycerol fermentation in *E. coli* have achieved production of a variety of chemicals, including 1,2-propanediol, ethanol, succinate, and lactate, among others. New kinetic and genome-scale *in silico* models have also revealed key information about flux control during glycerol utilization by *E. coli.*

This chapter presented the first experimental systems-level study of the anaerobic fermentation of glycerol in *E. coli*, to the best of our knowledge. We expected to obtain insights on underlying cellular systems that are affected by glycerol fermentation and the culture conditions that enable it via high-throughput omics analysis. Comparison of cell growth and glycerol consumption between several strains (BW25113WT, BW25113 Δ*adhE*, BW25113 Δ*gldA*, and BW25113 Δ*adhE* Δ*gldA*) revealed stronger impairment of glycerol fermentation in the BW25113Δ*adhE* strain and allowed determination of sample harvest time-points for proteomic analysis. A combination of comparisons between the wild-type strain and the mutant with impaired glycerol utilization in conditions with and without glycerol availability revealed 78 proteins that were differentially expressed during glycerol fermentation. Identification of 27 of these proteins by MS analysis and gel matching to online databases powered the development of hypotheses about differentially expressed proteins and their involvement in glycerol fermentation.

As was expected, several proteins involved in glycerol dissimilation pathways were differentially expressed in our study. The dihydroxyacetone kinase subunit M (*dhaM*), subunit B from the glycerol dehydrogenase complex (*glpB*), and glyoxalase I (*gloA*) participate in glycerol conversion to DHAP and methylglyoxal conversion to lactate, respectively. Their levels of

differential expression suggested that the mutant with impaired glycerol utilization changes carbon flux distribution at the DHAP node, and not being able to meet redox and ATP requirements make glycerol consumption almost negligible. A predicted oxidoreductase (*ucpA*) was also differentially expressed during glycerol fermentation. This predicted oxidoreductase could potentially be catalyzing one of the steps in the conversion of methylglyoxal to 1,2-propanediol. Further enzyme activity assays could help support this hypothesis. Proteins Tpx and Eno also resulted with differences in expression. It is thought that the highly redox and PEP-limited environment propitiated by glycerol fermentation is the main cause for differential expression of both proteins.

Two outer membrane porins, OmpF and PhoE (OmpE), were also differentially expressed in our proteomic analysis. While *ompF* expression has been reported to be affected by expression of the *glpFKX* operon, PhoE is known to be regulated by phosphate levels in the medium. The *glpFKX* is expected to be expressed during glycerol fermentation and could be the explanation for *ompF* downregulation. As for PhoE, phosphate limitation in the medium has been proven to be crucial for efficient glycerol fermentation in *E. coli* and the phosphate-limited medium used in our analysis could be the cause for changes in PhoE expression.

In addition to PhoE, 12 additional proteins (PurE, GlyA, GlpB, PoxB, AceA, CarA, RpsE, Eno, NusA, DapD, FabH, and DeoD) indirectly regulated by the phosphate-sensitive regulator PhoB were differentially expressed during glycerol fermentation. Differences in two-component systems and proteins regulated by them were also observed. PhoP, the regulator of the two-component system PhoQ-PhoP was upregulated during glycerol fermentation. In addition, OmpF and TalB, were also differentially expressed and are regulated by two-component systems sensitive to osmolarity (OmpR-EnvZ) and catabolite regulation (CreB-CreC), respectively. Differential expression of these proteins may reveal a systems level effect of phosphate limitation during glycerol fermentation.

Other central pathways that were affected by glycerol fermentation were amino acid and nucleotide biosynthesis and degradation, and the AMC. Enzymes Mtn, FolP, GlyA, and AroC pticipate in pathways leading to methionine synthesis and degradation, and in the AMC. Disruption of several reactions revolving around these pathways suggest a network effect. In addition, FabH, was also differentially expressed. Fatty acid biosynthesis is linked to the AMC cycle in the conversion of S-adenosylmethionine to methylthioadenosine and N-acylhomoserine lactones. Several hypothesis revolve around the differential expression of these proteins: (1) AI-2 synthesis being affected by an increased pool of DHAP and G3P during

impaired glycerol fermentation and affecting other pathways involved in methionine biosynthesis and degradation, (2) increasing concentrations of acetate in the medium during glycerol fermentation affect methionine and homocysteine pools, (3) use of biosynthetic and degradation cycles as electron sinks during anaerobic glycerol utilization. Follow up experimental analyses will provide more information to support or discard these hypothesis and could provide valuable information about additional ways to improve glycerol fermentation in *E. coli.*

Changes in expression of amino acid biosynthetic and degradation cycles could be responsible for differential expression of the PepQ, a proline dipeptidase which hydrolyzes dipeptide substrates containing a proline residue at the carboxy-terminal. In addition, nucleotide biosynthesis was also affected as suggested by differential expression of PurE, DeoD, and CarA. Nucleotide biosynthesis and degradation have been previously reported to be affected during glycerol fermentation *in silico*. Contrary to what was originally thought, the cells might actually be using these energetically-expensive pathways to achieve redox balance during glycerol fermentation.

In addition to the abovementioned effects, upregulation of the magnesium-sensitive regulator PhoP and differential expression of seven proteins that use Mg^{2+} as a cofactor for activity or structure (AceA, Eno, EF-P, FolP, DapD, PoxB, and CarA) suggest magnesium-limited conditions during

glycerol metabolism. Differential expression of EF-P and RpsE, both involved in ribosome activity and structure, further support this hypothesis, since magnesium has been reported to be crucial for normal ribosomal structure. Media supplementation with different concentrations of magnesium could provide a basis for determination of the effect of glycerol fermentation on magnesium availability.

Although several promising hypotheses have arisen from the proteomic analysis of *E. coli* during glycerol fermentation, further experimental analyses are needed to support them. New insights provided by this proteomic analysis and by follow up experiments will power the continued improvement of glycerol fermentation in *E. coli.*

Chapter 6

Proteomic Analysis of Chinese hamster ovary cells under mild hypothermia

6.1. Introduction

Mammalian cell cultures are the dominant manufacturing system used for the production of biopharmaceutical products and drugs based on recombinant proteins due to their ability to develop processes for assembly, folding, and post-translational modifications (Lim et al., 2010). In the past two decades, the production of recombinant proteins for therapeutic purposes has grown immensely, but the demand for this growing market still surpasses production capacity (Wurm, 2004). Therefore, efficient mammalian cell systems with increased recombinant protein production are needed in order to meet market requirements.

Different culture parameters have been studied in order to increase culture viability and longevity and, subsequently, recombinant protein production. An approach that has been widely studied is the use of mild hypothermia, with temperatures ranging from 30-33°C. This approach has led to increases in production of recombinant proteins in batch cultures and has guided several systems level studies in CHO cells, which have reported improved productivities (Berrios et al., 2009; Fox et al., 2004; Schatz et al., 2003). Still, the underlying mechanisms for such a response under mild hypothermia are not understood with clarity. While all the reports to date have focused on metabolic changes in batch cultures, it is important to note that a clear mechanism will be very difficult to obtain from batch cultures, since temperature changes are linked to changes in specific growth rate and the two properties cannot be analyzed separately. Therefore, a different culture approach must be employed in order to characterize the effect of these culture parameters individually.

Chemostat (continuous) cultures offer the advantages of environmental control, reproducibility, and maintaining constant physicochemical conditions by allowing control of the specific growth rate of the cells (μ) through the dilution rate (D=F/R, where F=Feed flow and R=Reaction volume). In this way, chemostat cultures provide a viable option

to characterize cell behavior studying the effects of one variable at a time (Hoskisson and Hobbs, 2005).

Vergara *et al* (Vergara et al., 2014) recently performed a study with the goal of separately assessing the effect of mild hypothermia and specific growth rate on the production of recombinant human tissue plasminogen activator (rht-PA) produced by CHO cells, by comparing cultures grown at two different dilution rates (0.012 and 0.017hr^{-1}) and two different temperatures each (37 and 33°C). Cell viability and rht-PA production are shown in Figure 6.1.

In their study, rht-PA production was increased at the lower dilution rate (0.012hr^{-1}). In collaboration with the Altamirano research group (PUCV, Chile), CHO cell samples corresponding to the 0.012hr-1 dilution rate and grown at 33 and 37°C were analyzed by two-dimensional gel electrophoresis in order to elucidate which mechanisms are activated under mild hypothermia with the ultimate goal of engineering more efficient CHO cell systems for the production of recombinant proteins.

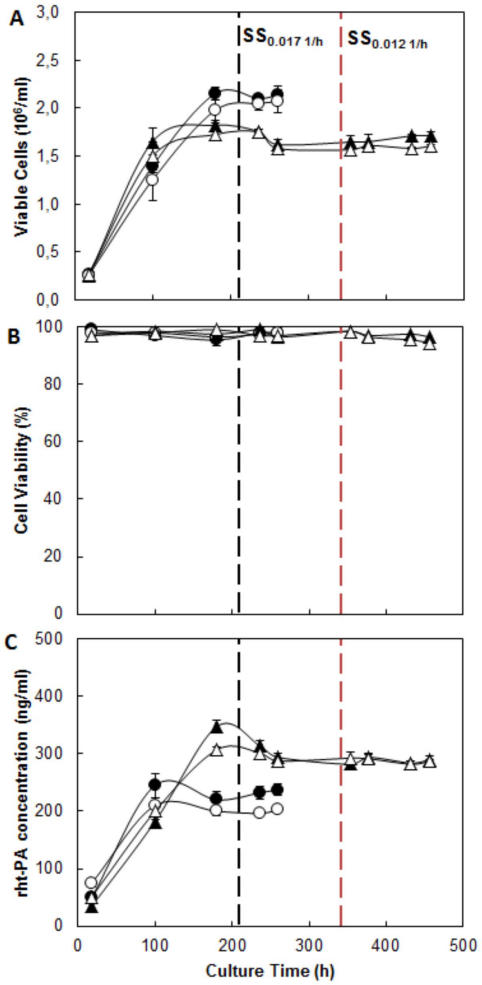


Figure 6.1. Profile of CHO cell growth, viability, and rht-PA production at different culture temperatures and dilution rates. (A) Viable cell concentration, (B) viability percentage, (C) rht-PA concentration. Circles, 0.017hr^{-1}; Triangles, 0.012hr^{-1}; Shaded, 37°C; Light, 33°C. Black dotted line: start of steady state (SS) for 0.017hr-1 cultures. Red dotted line: start of SS for 0.012hr^{-1} cultures (Vergara et al., 2014).

6.2. Materials and Methods

6.2.1. Cell line, culture medium and culture conditions

CHO cells (CHO TF 70R) were grown in continuous mode as reported by Vergara *et al* ((Vergara et al., 2014). Cells were harvested for proteomic

analysis after 4 residence times (333 hours for a dilution rate of 0.012hr^{-1}). At this time, more than 95% of the cells are at a defined physiological state and any change in their behavior must be due solely to changes in culture variables, e.g. temperature.

6.2.2. Proteomic analysis

6.2.2.1. Protein Extraction, Precipitation and Quantification

Cells were resuspended in 1.2mL lysis buffer as detailed in the Materials and Methods chapter (Chapter 3) and lysed by passing through a 25 gauge needle attached to a 1mL syringe. Samples were then centrifuged to remove cell debris and the supernatant was saved in 1.5mL vials. The resulting samples exhibited very high viscosity, and therefore, additional purification steps were investigated. A high concentration of nucleic acids has been shown to increase sample viscosity and could cause processing problems, such as background smears in silver-stained gels (GE Healthcare, 2010). In order to prevent further complications, samples were sonicated to shear nucleic acids into smaller fragments that would not interfere with isoelectric focusing. Samples were sonicated at an output of 2 and 10% duty for 5 minutes in a Branson Sonifier 250 (Branson Ultrasonics Co., Danbury, CT). Sonicated samples were then centrifuged at 12000RPM, 4°C for 10 minutes and the supernatant was saved for purification and analysis.

Precipitation, quantification and 2D gel electrophoresis were performed as presented in the Materials and Methods chapter (Chapter 3).

6.3. Results and Discussion

6.3.1. Proteomic analysis of CHO cells under mild hypothermia

Differential protein expression between two CHO cell culture conditions was studied in order to identify key proteins involved in the response of CHO cells to mild hypothermia and characterize the effects that lead to an increase in rht-PA production. The relationship between specific growth rate and specific productivity has been suggested to be dependent on the cell line, r-protein, limiting nutrients in the culture, and on culture temperature. A previous study by Vergara, *et al*. reported differences in rht-PA production by CHO cells (CHO TF 70R) due to changes in culture temperature and dilution rate (Vergara et al., 2014). From this study, culture conditions corresponding to a dilution rate of $0.012hr^{-1}$ were chosen for analysis to compare protein expression at 33°C and 37°C when they had passed 4 residence times. Although no major changes in metabolism (glucose and glutamate consumption, and lactate production) were observed between the two temperatures at a dilution rate of $0.012hr^{-1}$, a slightly higher average production of rht-PA was observed under mild hypothermia, although not statistically significant (Vergara et al., 2014). These results are in agreement

with previous reports about CHO cells in batch cultures that suggest better post-translational modifications at lower temperatures and lower specific growth rates (Kou et al., 2011). In order to identify key proteins, a proteomic analysis was performed as previously described in the Materials and Methods chapter (Chapter 3).

Twenty-three proteins were differentially expressed ≥ 2 fold (Figure 6.2). Eight of these proteins, exhibiting larger abundance differences and lower p-values, were extracted from coommassie blue-stained gels and identified by MS analysis and are labeled in Figure 6.2 and identified in Table 6.1. Due to lack of a complete genetic database for CHO cells, some proteins were identified using the National Center for Biotechnology Information (NCBI) database for 'animals' and resulted in inaccurate identification, being attributed to other organisms. Further BLAST analysis provided matching proteins from *Cricetulus griseus* (Chinese hamster) with high identity %.

Table 6.1. Differentially expressed proteins under mild hypothermia.

Spot No.	Protein ID (MS analysis)	Fold Difference	p-value
1	Calreticulin	+2.1	0.0232
2	PREDICTED: Chain A, Solution Structure Of Reduced Microsomal Rat Cytochrome B5, Nmr, Minimized Average Structure	-2.3	0.0056
3	PREDICTED: cytochrome b5	-2.7	0.1870
4	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	-2.5	0.1330
5	60 kDa heat shock protein	+2.0	0.1330
6	PREDICTED: osteoclast-stimulating factor 1-like	-1.9	0.0450
7	PREDICTED: stAR-related lipid transfer protein 4-like	-1.9	0.0470
8	hypothetical protein I79_005357	-3.1	0.1940

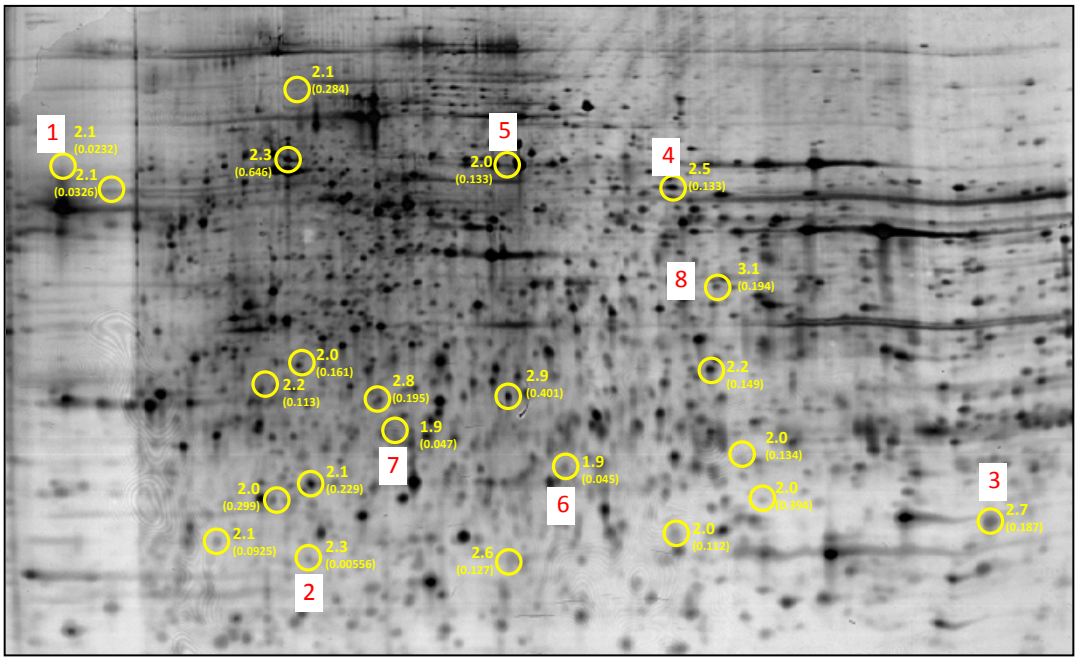


Figure 6.2. Differentially expressed proteins of CHO cells during mild hypothermia. 2D gel of CHO cells at 37°C and 0.012hr^{-1}. Circles show spots that were differentially expressed with the fold change and p-value in parenthesis. Number labels represent the eight proteins that were extracted from coommassie blue-stained gels and identified by MS (see Table 6.1).

The identified proteins had fold-differences $\geq$ 2 and p-values lower than 0.2. Protein spots with p-values higher than 0.05 (the usual threshold in proteomic analysis) were chosen due to their abundance in coommassie blue-stained gels and the magnitude of their fold change. A description of the biological functions of these proteins, and hypotheses about their role in the cellular response to mild hypothermia are presented in the following section.

6.3.2. Cellular systems affected by mild hypothermia

The underlying roles of the differentially expressed proteins on cellular metabolism and their potential relationships to mild hypothermia were investigated in order to guide further studies and engineering strategies. Although a relatively small number of proteins have been identified so far in this study, there are distinct relations between them, mainly their involvement in secretion pathways, including chaperones and proteins involved in Ca^{2+} homeostasis, and proteins involved in electron transfer (Figure 6.3).

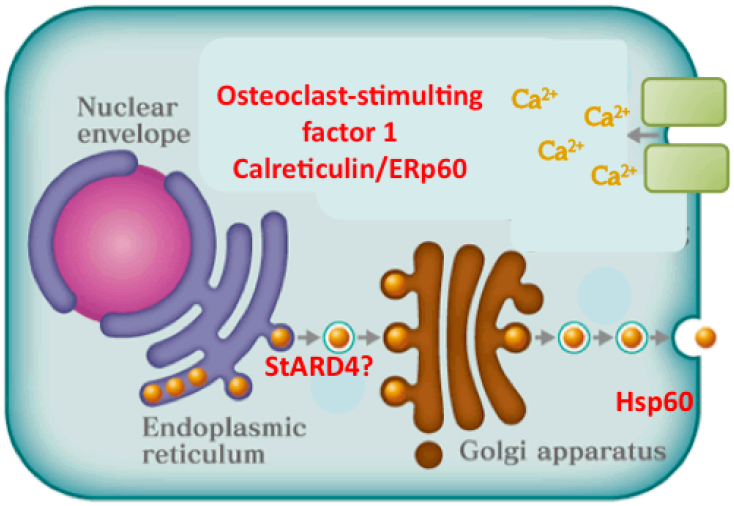


Figure 6.3. Differentially expressed proteins during mild hypothermia in CHO cells potentially involved in secretion pathways. The osteoclast-stimulating factor 1, calreticulin, Hsp60 are involved in pathways surrounding protein secretion. StARD4 may be involved in transport of proteins from the endoplasmic reticulum to the Golgi apparatus.

A desired trait in the production of therapeutic proteins in mammalian cell cultures is secretion into the medium, since this leads to simpler

purification and more economically viable downstream processing. Secretory mechanisms are therefore key in the production of recombinant proteins. Four of the seven proteins that were identified as being differentially expressed (Calreticulin/ERp60, osteoclast-stimulating factor 1, StARD4, and Hsp60) are potentially related to secretory pathways in CHO cells.

Calreticulin, also known as endoplasmic reticulum resident protein 60 (ERp60), was upregulated under mild hypothermia. Calreticulin has been linked to many functions, but mainly to (1) chaperoning and (2) regulating Ca^{2+} homeostasis (Chung et al., 2004; Michalak et al., 1999). Alterations in intracellular Ca^{2+} levels regulate a variety of cellular functions, including secretion, cytoplasmic and mitochondrial metabolism, and protein synthesis, modification, and folding, among others (Pozzan et al., 1994). Differential expression of calreticulin/ERp60 during mild hypothermia could reflect changes in protein synthesis, modification, and secretion due to the slightly higher production of recombinant protein during mild hypothermia. Another protein with altered protein expression is also involved in controlling Ca^{2+} levels in the cells. The osteoclast-stimulating factor 1 is well known to be crucially involved in the development of osteoclast cells which control the dissolution of calcium hydroxyapatite in vertebrates (Mueller et al., 2012; Roodman, 1996). Changes in levels of protein have been reported for osteoclast-stimulating factor 1 under Ca^{2+} limited conditions. Differential

expression of these two proteins during mild hypothermia may indicate unbalanced levels of Ca^{2+} in the cells during production of rht-PA. Since Ca^{2+} has been reported to be important for secretion processes in the cell (Coorssen et al., 1996), which are key for the production of recombinant proteins. Ca^{2+} supplementation to the culture media could reveal the effects of this ion on recombinant protein production and provide more information to confirm this hypothesis.

The StAR-related lipid transfer protein 4 (StARD4) was also differentially expressed under mild hypothermia. Steroidogenic acute regulatory (StAR) proteins are soluble protein carriers that mediate the intracellular transport of hydrophobic lipids (Liscum and Munn, 1999). StAR-related lipid transfer proteins (StART) are a family of proteins with homology to StAR, also containing a lipid-binding domain. Another member of the StART family, the ceramide transfer protein (CERT), is a key player in secretory transport (Hanada et al., 2003). CERT mediates the ATP-dependent transport of ceramide from the endoplasmic reticulum (ER) to the Golgi complex. A recent study investigating the effect of a CERT mutant with increased ceramide transfer activity on the production of the therapeutic protein HSA, reported an increase in HSA titer in chemically defined fed-batch processes (Florin et al., 2009). The study demonstrated that the level of expression and activity of this key molecule, regulating transport from the ER

to the plasma membrane via the trans-Golgi network, can significantly influence yields in CHO cell culture production processes. Differential expression of StARD4 in rht-PA-producing cells during mild hypothermia could suggest a similar effect of this protein on secretory mechanisms in CHO cells.

The 60kDa heat shock protein (Hsp60) was also differentially expressed (+2.0 fold change) under mild hypothermia. Hsp60 is a major molecular chaperone, which functions in protein folding and the assembly of oligomeric proteins (Ellis and Vandervies, 1991). Hsp60 has been established as a component in bacterial protein secretion and transport across the plasma membrane and suggested to have similar activity in CHO cells (Soltys and Gupta, 1997). Calreticulin and Hsp60 have been previously reported as differentially expressed in a study using transcriptomics and proteomics to investigate temperature changes in batch CHO cell cultures with the goal of identifying targets for increasing the production of erythropoietin (Baik et al., 2006).

Simultaneous differential expression of ERp60, osteoclast-stimulating factor 1, StARD4, and Hsp60 could imply alterations in secretory mechanisms in CHO cells during mild hypothermia. Due to the multifunctionality of some of these proteins, other processes, such as chaperoning and maintaining Ca^{2+} homeostasis could also become affected. Although additional experiments are

needed in order to elucidate regulatory mechanisms, this data suggests that reported increases in yield and productivity of therapeutic proteins in CHO cell batch cultures during mild hypothermia (Becerra et al., 2012; Fox et al., 2004; Marchant et al., 2008; Schatz et al., 2003) could result from changes in secretory pathways.

Two other proteins identified as being differentially expressed are proteins involved in oxidoreductase reactions, suggesting changes in redox potential and electron transport during mild hypothermia. Cytochrome b5, which was downregulated during mild hypothermia, was represented by more than one spot (spots 2 and 3) in the two dimensional gel. Cytochrome b5 is involved in electron transfer in a number of oxidative reactions in biological tissues, such as metabolism of fats and steroids (Schenkman and Jansson, 2003). Cytochrome b5 forms a complex with cytochrome P450, and acts as a component in many reactions with NADPH-cytochrome P450 reductase and NADH cytochrome b5 reductase, functioning as an electron donor (Schenkman and Jansson, 2003). On the other hand, the dihydrolipoyllysine-residue succinyltransferase component of the 2-oxoglutarate dehydrogenase complex was also downregulated under mild hypothermia. The 2-oxoglutarate dehydrogenase complex is key for amino acid degradation pathways and for the generation of precursor metabolites and energy. Even though no drastic changes were observed in glucose and

glutamate consumption, and lactate production in the analyzed samples (Vergara et al., 2014), in contrast to data observed for batch cultures in previous studies (Berrios et al., 2009), lower expression of 2-oxoglutarate dehydrogenase could represent a shift in central metabolic routes for the production of recombinant protein.

Carbon metabolism and redox balance have been reported in omics analysis as affected by environmental changes in recombinant CHO and NS0 (mouse myeloma) cells (Seth et al., 2007b; Yee et al., 2008). Proteins involved in redox reactions and electron transport, including Cytochrome b5, were differentially expressed in a combined transcriptomic and proteomic study analyzing 11 lines of NS0 cells with different antibody productivities. In addition, a proteomic study of CHO and mouse hybridoma (MAK) cells under butyrate addition revealed differential expression of eight proteins involved in cytosol and endoplasmic reticulum redox control. In both studies, the additional differential expression of thioredoxin, protein disulfide isomerases, and proteins involved in folding processes suggests that changes in cellular redox balance may be disturbing protein processing by affecting chaperones and folding catalysts. These protein processing mechanisms have been suggested to play important roles in the production of recombinant proteins (Seth et al., 2007a).

Lastly, one of the differentially expressed proteins in response to culture temperature in continuous CHO cell cultures was identified as a hypothetical protein. Although there is not sufficient data from this proteomic analysis to support a hypothesis about the function of this protein, additional omics experiments comparing more culture conditions should help elucidate its function and role under mild hypothermia.

These results confirm the importance of secretion mechanisms and overall redox balance in the cells for the production of recombinant proteins and provide more specific targets for further investigation and engineering for the increased productivity of recombinant proteins during mild hypothermia.

6.4. Conclusions

Mild hypothermia has been extensively studied in terms of improving productivity and yield of recombinant proteins in batch cultures, but the inevitable connectivity between temperature and specific growth rate makes it impossible to pinpoint an observed physiological behavior to the action of a particular variable. In order to better understand the effects of mild hypothermia on CHO cell metabolism, a proteomic analysis was performed to detect differences in protein expression between cultures grown at 33 and 37°C and a dilution rate of 0.012hr^{-1}.

Eight differentially expressed proteins were identified and their roles were assessed in terms of the effect of a lower culture temperature, revealing two main systems that were affected by mild hypothermia: secretion and oxidoreductive processes. Ca^{2+} homeostasis was also highlighted in some of these proteins, as it is crucial for secretion processes. Although follow up experiments should provide additional evidence to support this hypothesis, the differential expression of proteins related to secretion mechanisms (Calreticulin/ERp60, osteoclast-stimulating factor 1, StARD4, and Hsp60) suggests that this key mechanism in the production of recombinant proteins is being affected by the culture temperature and may be responsible for the observed increases of recombinant protein yields in batch cultures. In addition, the differential expression of Cytochrome b5 and 2-oxoglutarate dehydrogenase complex suggested alterations in redox balances in the cells. Previous omics studies in mammalian cells with improved productivities of recombinant proteins have also reported alteration of proteins involved in redox control and, moreover, in protein processing(Seth et al., 2007b; Yee et al., 2008). The simultaneous differential expression of these two systems suggests a link between redox imbalances and protein folding mechanisms, both of which are key for the production of recombinant proteins (Seth et al., 2007a). An additional differentially expressed protein was identified as a hypothetical protein. Although there is not sufficient information to attribute

a specific role to this protein, due to results from this analysis, its possible involvement in secretion mechanisms in the cell should be the focus of initial studies to reveal its function.

Although valuable information was obtained from the proteomic analysis of CHO cells in mild hypothermia, there are some limitations that must be overcome in order to gain additional insights. The limited availability of genetic information for CHO cells leading to limited protein databases made identification of more proteins difficult. Data from MALDI/TOF MS analysis were compared to the NCBI 'Animal' database and further BLAST analysis was needed in order to obtain more accurate identification of most of the proteins. The continued improvement of these databases will make proteomics an even stronger tool for understanding CHO cell metabolism.

In addition to the proposed future work, protein changes with respect to variations in dilution rate should also be assessed using proteomic analysis. This should provide additional information about which systems are being altered by another individual variable and, in conjunction with the information obtained from this work, could help map physiological behavior according to culture variables and could guide engineering efforts depending on desired cellular properties.

Chapter 7

Concluding Remarks

Thanks to the advances of the genomic revolution, a significant number of genomes have been sequenced and numerous genetic tools continue to be developed. Metabolic and cellular engineering have consequently become robust approaches for the development of biofactories, consisting of microbial or mammalian cell cultures engineered to produce molecules with desired traits for therapeutic or industrial applications. Furthermore, advances in systems biology have yielded powerful tools for the analysis of thousands of cellular components via functional genomics. Among functional genomics, proteomics presents a powerful tool to analyze the entire set of proteins, characterize the role of key protein targets, and engineer cellular systems to achieve improved properties.

The response of *Escherichia coli* to short-chain fatty acids (SCFAs) was assessed via proteomic analysis. Given their simple and easy-to-manipulate

chemical structures, SCFAs are valuable feedstocks for many industrial applications, but their antimicrobial properties create a limiting factor in the design of industrial strains with high SCFA productivity and yields. While the microbial production of SCFAs by engineered *Escherichia coli* has been demonstrated recently, productivity and yields are still limited by these antimicrobial properties.

Limited production of the desired chemicals and reduced tolerance to toxic metabolites, including the desired products, are among the obstacles that can be encountered throughout the process of engineering microbial strains. Overcoming these obstacles begins by adequately characterizing and understanding the inhibition and production mechanisms of potential microorganisms for the production of the desired chemicals.

The findings reported in this work confirmed the advantages of omic analyses on guiding strategies to address toxicity challenges in microorganisms. The comparative proteomic analysis of wild-type *E. coli* MG1655 under octanoic acid (C8) stress (15mM) and its reference condition (0mM) revealed differential expression of proteins involved in transport and structural roles, oxidative stress, protein synthesis, and metabolic functions. Additional assays involving deletion and overexpression mutants suggested that membrane damage and oxidative stress are the main routes of inhibition by SCFAs. Furthermore, the differential expression of additional membrane

proteins detected by SDS-PAGE reinforced the hypothesis of a strong membrane involvement in the response of *E. coli* to SCFAs. From among these proteins, the outer membrane porin OmpF had the greatest impact on SCFA tolerance and was proposed as facilitating SCFA transport into the cells. One area that has not yet been exploited for tolerance of SCFAs is the study of FA exporters in *E. coli.* In this context, outer membrane porins that have stood out in this study should be further investigated, and the potential of engineering these targets at the protein structure level should be analyzed.

The fermentative metabolism of glycerol in *E. coli* was also studied from a systems perspective. Glycerol has gained increased attention as a substrate for biorefineries in recent years, due to an increase in the production of biodiesel and bioethanol, both of which co-produce glycerol as a by-product. Its high degree of reduction also makes it a valuable carbon source for the production of reduced chemicals. While substantial work has been done to understand, characterize and exploit the potential of glycerol as a feedstock for the microbial production of a wide range of chemicals, they have followed a bottom-up approach, focusing on engineering specific enzymes and pathways. This chapter presented the first experimental systems-level study of the anaerobic fermentation of glycerol in *E. coli.* Comparison of cell growth and glycerol consumption between several strains (BW25113WT, BW25113 Δ*adhE*, BW25113 Δ*gldA*, and BW25113 Δ*adhE*

Δ*gldA*) revealed stronger impairment of glycerol fermentation in BW25113Δ*adhE.* Simultaneous comparisons between the wild-type strain and the mutant with impaired glycerol utilization in glycerol-fermenting and non glycerol-fermenting states revealed 78 proteins that were differentially expressed during glycerol fermentation. Identification of 27 of these proteins by MS analysis and gel matching to online databases powered the development of hypotheses about differentially expressed proteins and their involvement in glycerol fermentation.

As was expected, several proteins involved in glycerol dissimilation pathways were differentially expressed in our study. Gene deletion experiments and enzyme activity assays may provide additional information to link a particular function to this protein. Other proteins that were differentially expressed in this study had functions including outer membrane porins, amino acid and nucleotide biosynthesis and degradation, and proteins responding to phosphate and magnesium limitations. Although several promising hypotheses have arisen from the proteomic analysis of *E. coli* during glycerol fermentation, further experimental analyses are needed to support them. New insights provided by this proteomic analysis and by follow up experiments will power the continued improvement of glycerol fermentation in *E. coli.*

A proteomic analysis of CHO cells under mild hypothermia was also investigated in this thesis. CHO cell cultures are widely used for the production of recombinant proteins due to their ability to develop processes for correct assembly, folding and post-translational modifications. Several studies in CHO cell batch cultures have identified mild hypothermia (culture temperatures between 30-33°C) as one of the variables responsible for increasing the production of recombinant proteins. This variable has been studied in batch cultures, but the inability to uncouple culture temperature and specific cell growth under this culture condition has made it difficult to assess specific gene or protein targets responsible for the increased productivity. In order to better understand the effects of mild hypothermia on CHO cell metabolism, a proteomic analysis was performed to detect differences in protein expression between cultures grown at 33 and 37°C and a dilution rate of 0.012hr^{-1}. Eight differentially expressed proteins were identified and their roles were assessed in terms of the effect of a lower culture temperature, revealing two main systems that were affected by mild hypothermia: secretion and oxidoreductive processes. Ca^{2+} homeostasis was also highlighted in some of these proteins, as it is crucial for secretion processes. Although follow up experiments should provide additional evidence to support this hypothesis, the differential expression of proteins related to secretion mechanisms suggests that this key mechanism in the

production of recombinant proteins is being affected by the culture temperature and may be responsible for the observed increases of recombinant protein yields in batch cultures. In addition, the differential expression proteins involved in redox balance may suggest that protein folding mechanisms are being affected. An additional differentially expressed protein was identified as a hypothetical protein. Although there is not sufficient information to attribute a specific role to this protein, due to results from this analysis, its possible involvement in secretion mechanisms in the cell should be the focus of initial studies to reveal its function.

In addition to the proposed future work, protein changes with respect to variations in dilution rate should also be assessed using proteomic analysis. This should provide additional information about which systems are being altered by another individual variable and, in conjunction with the information obtained from this work, could help map physiological behavior according to culture variables and could guide engineering efforts depending on desired cellular properties.

This thesis presented the use of proteomic analysis to characterize different aspects of metabolism with the ultimate goal of developing more efficient platforms for chemicals, biofuels, and biopharmaceutical applications. The work described in this thesis has provided a strong foundation towards achieving the ultimate goal.

References

Alemoham.Mm, Knowles, C. J., 1974. Osmotically induced volume and turbidity changes of *Escherichia coli* due to salts, sucrose and glycerol, with particular reference to raid permeation of glycerol into cell. Journal of General Microbiology. 82**,** 125-142.

Alper, H., Moxley, J., Nevoigt, E., Fink, G. R., Stephanopoulos, G., 2006. Engineering yeast transcription machinery for improved ethanol tolerance and production. Science. 314**,** 1565-1568.

Alsaker, K. V., Papoutsakis, E. T., 2005. Transcriptional program of early sporulation and stationary-phase events in *Clostridium acetobutylicum*. Journal of Bacteriology. 187**,** 7103-7118.

Alsaker, K. V., Spitzer, T. R., Papoutsakis, E. T., 2004. Transcriptional analysis of spo0A overexpression in *Clostridium acetobutylicum* and its effect on the cell's response to butanol stress. Journal of Bacteriology. 186**,** 1959-1971.

Altintas, M. M., Eddy, C. K., Zhang, M., McMillan, J. D., Kompala, D. S., 2006. Kinetic modeling to optimize pentose fermentation in *Zymomonas mobilis*. Biotechnology and Bioengineering. 94**,** 273-295.

Amin, M., Kothamachu, V. B., Feliu, E., Scharf, B. E., Porter, S. L., Soyer, O. S., 2014. Phosphate sink containing two-component signaling systems as tunable threshold devices. Plos Computational Biology. 10.

Association, E. I., Annual Energy Outlook 2009 with Projections to 2030. In: Energy, U. D. o., (Ed.), 2009.

Atsumi, S., Hanai, T., Liao, J. C., 2008. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. Nature. 451**,** 86-U13.

Atsumi, S., Liao, J. C., 2008. Metabolic engineering for advanced biofuels production from *Escherichia coli*. Current Opinion in Biotechnology. 19**,** 414-419.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., Mori, H., 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Molecular Systems Biology. 2.

Baez-Viveros, J. L., Osuna, J., Hernandez-Chavez, G., Soberon, X., Bolivar, F., Gosset, G., 2004. Metabolic engineering and protein directed evolution increase the yield of L-phenylalanine synthesized from glucose in *Escherichia coli.* Biotechnology and Bioengineering. 87**,** 516-524.

Baik, J. Y., Lee, G. M., 2010. A DIGE Approach for the assessment of differential expression of the CHO proteome under sodium butyrate addition: Effect of Bcl-x(L) overexpression. Biotechnology and Bioengineering. 105, 358-367.

Baik, J. Y., Lee, M. S., An, S. R., Yoon, S. K., Joo, E. J., Kim, Y. H., Park, H. W., Lee, G. M., 2006. Initial transcriptome and proteome analyses of low culture temperature-induced expression in CHO cells producing erythropoietin. Biotechnology and Bioengineering. 93, 361-371.

Bailey, J. E., 1991. Toward a science of metabolic engineering. Science. 252, 1668-1675.

Bassilana, M., Damiano, E., Leblanc, G., 1984. Kinetic properties of Na^+-H^+ antiport in *Eschierichia coli* membrane vesicles - Effects of imposed electrical potential, proton gradient, and internal pH. Biochemistry. 23, 5288-5294.

Becerra, S., Berrios, J., Osses, N., Altamirano, C., 2012. Exploring the effect of mild hypothermia on CHO cell productivity. Biochemical Engineering Journal. 60, 1-8.

Beeston, A. L., Surette, M. G., 2002. *pfs*-dependent regulation of autoinducer 2 production in *Salmonella enterica* serovar Typhimurium. Journal of Bacteriology. 184, 3450-3456.

Bengtsson, O., Jeppsson, M., Sonderegger, M., Parachin, N. S., Sauer, U., Hahn-Hagerdal, B., Gorwa-Grauslund, M. F., 2008. Identification of common traits in improved xylose-growing *Saccharomyces cerevisiae* for inverse metabolic engineering. Yeast. 25, 835-847.

Bensmail, H., Haoudi, A., 2005. Data mining in genomics and proteomics. Journal of Biomedicine and Biotechnology. 63-64.

Berrios, J., Diaz-Barrera, A., Bazan, C., Altamirano, C., 2009. Relationship between tissue plasminogen activator production and specific growth rate in Chinese Hamster Ovary cells cultured in mannose at low temperature. Biotechnology Letters. 31, 1493-1497.

Black, P. N., 1990. Characterization of FadL-specific fatty acid binding in *Escherichia coli*. Biochimica Et Biophysica Acta. 1046, 97-105.

Black, P. N., Dirusso, C. C., 1994. Molecular and biochemical analyses of fatty acid transport, metabolism, and gene regulation in *Escherichia coli*. Biochimica Et Biophysica Acta-Lipids and Lipid Metabolism. 1210, 123-145.

Black, P. N., DiRusso, C. C., 2003. Transmembrane movement of exogenous long-chain fatty acids: Proteins, enzymes, and vectorial esterification. Microbiology and Molecular Biology Reviews. 67, 454-+.

Black, P. N., Said, B., Ghosn, C. R., Beach, J. V., Nunn, W. D., 1987. Purification and characterization of an outer membrane-bound protein involved in long-chain fatty acid transport in *Escherichia coli*. Journal of Biological Chemistry. 262, 1412-1419.

Blankschien, M. D., Clomburg, J., Gonzalez, R., 2010. Metabolic engineering of *Escherichia coli* for the production of succinate from glycerol. Metabolic Engineering. 12, 409-419.

Bocquetpages, C., Lazdunski, C., Lazdunski, A., 1981. Lipid synthesis-dependent biosynthesis (or assembly) of major outer membrane proteins of *Escherichia coli*. European Journal of Biochemistry. 118, 105-111.

Boenigk, R., Bowien, S., Gottschalk, G., 1993. Fermentation of glycerol to 1,3-propanediol in continuous cultures of *Citrobacter freundii*. Applied Microbiology and Biotechnology. 38, 453-457.

Bonomo, J., Lynch, M. D., Warnecke, T., Price, J. V., Gill, R. T., 2008. Genome-scale analysis of anti-metabolite directed strain engineering. Metabolic Engineering. 10, 109-120.

Booth, I. R., Glycerol and Methylglyoxal Metabolism. EcoSal Plus, 2005.

Booth, I. R., Ferguson, G. P., Miller, S., Li, C., Gunasekera, B., Kinghorn, S., 2003. Bacterial production of methylglyoxal: a survival strategy or death by misadventure? Biochemical Society Transactions. 31, 1406-1408.

Borden, J. R., Papoutsakis, E. T., 2007. Dynamics of genomic-library enrichment and identification of solvent tolerance genes for *Clostfidium acetobutylicum*. Applied and Environmental Microbiology. 73**,** 3061-3068.

Bruno-Barcena, J. M., Azcarate-Peril, M. A., Hassan, H. M., 2010. Role of antioxidant enzymes in bacterial resistance to organic acids. Appl. Environ. Microbiol. 76**,** 2747-2753.

Brynildsen, M. P., Liao, J. C., 2009. An integrated network approach identifies the isobutanol response network of *Escherichia coli*. Molecular Systems Biology. 5.

Burgard, A. P., Pharkya, P., Maranas, C. D., 2003. OptKnock: A bilevel programming framework for identifying gene knockout strategies for microbial strain optimization. Biotechnology and Bioengineering. 84**,** 647-657.

Butler, M., Meneses-Acosta, A., 2012. Recent advances in technology supporting biopharmaceutical production from mammalian cells. Applied Microbiology and Biotechnology. 96**,** 885-894.

Buzzai, M., Bauer, D. E., Jones, R. G., DeBerardinis, R. J., Hatzivassiliou, G., Elstrom, R. L., Thompson, C. B., 2005. The glucose dependence of Akt-transformed cells can be reversed by pharmacologic activation of fatty acid beta-oxidation. Oncogene. 24**,** 4165-4173.

Carmel-Harel, O., Storz, G., 2000. Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. Annual Review of Microbiology. 54**,** 439-461.

Cha, M. K., Kim, I. H., 1999. "25-kDa thiol peroxidase" (TPx II) acts as "housekeeping" antioxidant. Journal of Biochemistry and Molecular Biology. 32**,** 506-510.

Cha, M. K., Kim, W. C., Lim, C. J., Kim, K., Kim, I. H., 2004. *Escherichia coli* periplasmic thiol peroxidase acts as lipid hydroperoxide peroxidase and the principal antioxidative function during anaerobic growth. Journal of Biological Chemistry. 279**,** 8769-8778.

Chagneau, C., Heyde, M., Alonso, S., Portalier, R., Laloi, P., 2001. External-pH-dependent expression of the maltose regulon and *ompF* gene in *Escherichia coli* is affected by the level of glycerol kinase, encoded by *glpK*. Journal of Bacteriology. 183**,** 5675-5683.

Chandran, V., Luisi, B. F., 2006. Recognition of enolase in the *Escherichia coli* RNA degradosome. Journal of Molecular Biology. 358**,** 8-15.

Charles, I. G., Lamb, H. K., Pickard, D., Dougan, G., Hawkins, A. R., 1990. Isolation, characterization and nucleotide-sequences of the *aroC* genes encoding chorismate synthase from *Salmonella typhi* and *Escherichia coli*. Journal of General Microbiology. 136**,** 353-358.

Chatzifragkou, A., Papanikolaou, S., Dietz, D., Doulgeraki, A. I., Nychas, G.-J. E., Zeng, A.-P., 2011. Production of 1,3-propanediol by *Clostridium butyricum* growing on biodiesel-derived crude glycerol through a non-sterilized fermentation process. Applied Microbiology and Biotechnology. 91**,** 101-112.

Chen, D. C., Liu, Z. Q., Ma, X. B., Hua, D., 2005. Selecting genes by test statistics. Journal of Biomedicine and Biotechnology. 132-138.

Cheng, J. S., Qiao, B., Yuan, Y. J., 2008. Comparative proteome analysis of robust *Saccharomyces cerevisiae* insights into industrial continuous and batch fermentation. Applied Microbiology and Biotechnology. 81**,** 327-338.

Cheng, J. S., Zhou, X., Ding, M. Z., Yuan, Y. J., 2009. Proteomic insights into adaptive responses of *Saccharomyces cerevisiae* to the repeated vacuum fermentation. Applied Microbiology and Biotechnology. 83**,** 909-923.

Chiang, P. K., Gordon, R. K., Tal, J., Zeng, G. C., Doctor, B. P., Pardhasaradhi, K., McCann, P. P., 1996. S-adenosylmethionine and methylation. Faseb Journal. 10**,** 471-480.

Choi, W. J., Hartono, M. R., Chan, W. H., Yeo, S. S., 2011. Ethanol production from biodiesel-derived crude glycerol by newly isolated *Kluyvera cryocrescens*. Applied Microbiology and Biotechnology. 89**,** 1255-1264.

Chotani, G., Dodge, T., Hsu, A., Kumar, M., LaDuca, R., Trimbur, D., Weyler, W., Sanford, K., 2000. The commercial production of chemicals using pathway engineering. Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology. 1543**,** 434-455.

Chung, J. Y., Lim, S. W., Hong, Y. J., Hwang, S. O., Lee, G. M., 2004. Effect of doxycycline-regulated calnexin and calreticulin expression on specific thrombopoietin productivity of recombinant Chinese hamster ovary cells. Biotechnology and Bioengineering. 85**,** 539-546.

Cintolesi, A., Clomburg, J. M., Gonzalez, R., 2014. *In silico* assessment of the metabolic capabilities of an engineered functional reversal of the beta-oxidation cycle for the synthesis of longer-chain (C >= 4) products. Metabolic Engineering. 23**,** 100-115.

Cintolesi, A., Clomburg, J. M., Rigou, V., Zygourakis, K., Gonzalez, R., 2012. Quantitative analysis of the fermentative metabolism of glycerol in *Escherichia coli.* Biotechnology and Bioengineering. 109**,** 187-198.

Cipak, A., Jaganjac, M., Tehlivets, O., Kohlwein, S. D., Zarkovic, N., 2008. Adaptation to oxidative stress induced by polyunsaturated fatty acids in yeast. Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids. 1781**,** 283-287.

Clark, S. W., Bennett, G. N., Rudolph, F. B., 1989. Isolation and characterization of mutants of *Clostridium acetobutylicum* ATCC-824 deficient in acetoacetyl-coenzyme A - Acetate butyrate-coenzyme A-transferase (EC-2.8.3.9) and in other solvent pathway enzymes. Applied and Environmental Microbiology. 55**,** 970-976.

Clomburg, J., Understanding fermentative glycerol metabolism and its application for the production of fuels and chemicals. Chemical and Biomolecular Engineering, Vol. PhD. Rice University, 2012, pp. 189.

Clomburg, J. M., Gonzalez, R., 2011. Metabolic engineering of *Escherichia coli* for the production of 1,2-propanediol from glycerol. Biotechnology and Bioengineering. 108**,** 867-879.

Clomburg, J. M., Gonzalez, R., 2013. Anaerobic fermentation of glycerol: a platform for renewable fuels and chemicals. Trends in Biotechnology. 31**,** 20-28.

Cole, S. T., Eiglmeier, K., Ahmed, S., Honore, N., Elmes, L., Anderson, W. F., Weiner, J. H., 1988. Nucleotide sequence and gene polypeptide relationships of the *glpABC* operon encoding the anaerobic glycerol-3-phosphate dehydrogenase of *Escherichia coli* K-12. Journal of Bacteriology. 170**,** 2448-2456.

Coorssen, J. R., Schmitt, H., Almers, W., 1996. Ca^{2+}-triggers massive exocytosis in Chinese hamster ovary cells. Embo Journal. 15**,** 3787-3791.

Cornell, K. A., Swarts, W. E., Barry, R. D., Riscoe, M. K., 1996. Characterization of recombinant *Escherichia coli* 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase: Analysis of enzymatic activity and substrate specificity. Biochemical and Biophysical Research Communications. 228**,** 724-732.

Cronan, J. E., Thomas, J., 2009. Bacterial fatty acid synthesis and its relationships with polyketide synthetic pathways. Complex Enzymes in Microbial Natural Product Biosynthesis, Part B: Polyketides, Aminocoumarins and Carbohydrates. vol. 459, pp. 395-433.

Daniel, R., Stuertz, K., Gottschalk, G., 1995. Biochemical and molecular characterization of the oxidative branch of glycerol utilization by *Citrobacter freundii*. Journal of Bacteriology. 177**,** 4392-4401.

Daran-Lapujade, P., Jansen, M. L. A., Daran, J. M., van Gulik, W., de Winde, J. H., Pronk, J. T., 2004. Role of transcriptional regulation in controlling fluxes in central carbon metabolism of *Saccharomyces cerevisiae* - A chemostat culture study. Journal of Biological Chemistry. 279**,** 9125-9138.

Datsenko, K. A., Wanner, B. L., 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proceedings of the National Academy of Sciences of the United States of America. 97**,** 6640-6645.

Davis, M. S., Solbiati, J., Cronan, J. E., 2000. Overproduction of acetyl-CoA carboxylase activity increases the rate of fatty acid biosynthesis in *Escherichia coli.* Journal of Biological Chemistry. 275**,** 28593-28598.

Della Ragione, F., Porcelli, M., Carteni-Farina, M., Zappia, V., Pegg, A. E., 1985. *Escherichia coli* S-adenosylhomocysteine/5'-methylthioadenosine nucleosidase. Purification, substrate specificity and mechanism of action. The Biochemical journal. 232**,** 335-41.

Dellomonaco, C., Clomburg, J. M., Miller, E. N., Gonzalez, R., 2011. Engineered reversal of the beta-oxidation cycle for the synthesis of fuels and chemicals. Nature. 476**,** 355-U131.

Dellomonaco, C., Rivera, C., Campbell, P., Gonzalez, R., 2010. Engineered respiro-fermentative metabolism for the production of biofuels and biochemicals from fatty acid-rich feedstocks. Applied and Environmental Microbiology. 76**,** 5067-5078.

Desbois, A. P., 2012. Potential applications of antimicrobial fatty acids in medicine, agriculture and other industries. Recent patents on anti-infective drug discovery. 7**,** 111-22.

Desbois, A. P., Smith, V. J., 2010. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. Applied Microbiology and Biotechnology. 85**,** 1629-1642.

Deutscher, J., Francke, C., Postma, P. W., 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. Microbiology and Molecular Biology Reviews. 70**,** 939-+.

Dharmadi, Y., Gonzalez, R., 2004. DNA microarrays: Experimental issues, data analysis, and application to bacterial systems. Biotechnology Progress. 20**,** 1309-1324.

Dharmadi, Y., Murarka, A., Gonzalez, R., 2006. Anaerobic fermentation of glycerol by *Escherichia coli*: A new platform for metabolic engineering. Biotechnology and Bioengineering. 94**,** 821-829.

Ding, M. Z., Cheng, J. S., Xiao, W. H., Qiao, B., Yuan, Y. J., 2009. Comparative metabolomic analysis on industrial continuous and batch ethanol fermentation processes by GC-TOF-MS. Metabolomics. 5**,** 229-238.

Dinh, T. N., Nagahisa, K., Yoshikawa, K., Hirasawa, T., Furusawa, C., Shimizu, H., 2009. Analysis of adaptation to high ethanol concentration in *Saccharomyces cerevisiae* using DNA microarray. Bioprocess and Biosystems Engineering. 32**,** 681-688.

DiRusso, C. C., Black, P. N., Weimar, J. D., 1999. Molecular inroads into the regulation and metabolism of fatty acids, lessons from bacteria. Progress in Lipid Research. 38**,** 129-197.

Doerfel, L. K., Wohlgemuth, I., Kothe, C., Peske, F., Urlaub, H., Rodnina, M. V., 2013. EF-P Is Essential for rapid synthesis of proteins containing consecutive proline residues. Science. 339**,** 85-88.

Doi, H., Hoshino, Y., Nakase, K., Usuda, Y., 2014. Reduction of hydrogen peroxide stress derived from fatty acid beta-oxidation improves fatty acid utilization in *Escherichia coli*. Applied Microbiology and Biotechnology. 98**,** 629-639.

Donahue, J. L., Bownas, J. L., Niehaus, W. G., Larson, T. J., 2000. Purification and characterization of *glpX*-encoded fructose 1,6-bisphosphatase, a new enzyme of the

glycerol 3-phosphate regulon of *Escherichia coli.* Journal of Bacteriology. 182**,** 5624-5627.

Duarte, N. C., Palsson, B. O., Fu, P. C., 2004. Integrated analysis of metabolic phenotypes in *Saccharomyces cerevisiae*. BMC Genomics. 5.

Durnin, G., Clomburg, J., Yeates, Z., Alvarez, P. J. J., Zygourakis, K., Campbell, P., Gonzalez, R., 2009. Understanding and harnessing the microaerobic metabolism of glycerol in *Escherichia coli*. Biotechnology and Bioengineering. 103**,** 148-161.

Edwards, J. S., Ibarra, R. U., Palsson, B. O., 2001. *In silico* predictions of *Escherichia coli* metabolic capabilities are consistent with experimental data. Nature Biotechnology. 19**,** 125-130.

Ellis, R. J., Vandervies, S. M., 1991. Molecular chaperones. Annual Review of Biochemistry. 60**,** 321-347.

EPA, Renewable Fuels Standard. 2010.

EPA, National Greenhouse Gas Emissions Data. 2014.

Escalante, A., Salinas Cervantes, A., Gosset, G., Bolivar, F., 2012. Current knowledge of the *Escherichia coli* phosphoenolpyruvate-carbohydrate phosphotransferase system: peculiarities of regulation and impact on growth and product formation. Applied Microbiology and Biotechnology. 94**,** 1483-1494.

Espinosa-Gonzalez, I., Asomaning, J., Mussone, P., Bressler, D. C., 2014. Two-step thermal conversion of oleaginous microalgae into renewable hydrocarbons. Bioresource Technology. 158**,** 91-97.

Fajardo, D. A., Cheung, J., Ito, C., Sugawara, E., Nikaido, H., Misra, R., 1998. Biochemistry and regulation of a novel *Escherichia coli* K-12 porin protein, OmpG, which produces unusually large channels. Journal of Bacteriology. 180**,** 4452-4459.

Fay, J. P., Farias, R. N., 1975. Inhibitory action of fatty acids on growth of *Escherichia coli*. Journal of General Microbiology. 91**,** 233-240.

Fink, R. C., Scandalios, J. G., 2002. Molecular evolution and structure-function relationships of the superoxide dismutase gene families in angiosperms and their relationship to other eukaryotic and prokaryotic superoxide dismutases. Archives of Biochemistry and Biophysics. 399**,** 19-36.

Florin, L., Pegel, A., Becker, E., Hausser, A., Olayioye, M. A., Kaufmann, H., 2009. Heterologous expression of the lipid transfer protein CERT increases therapeutic protein productivity of mammalian cells. Journal of Biotechnology. 141**,** 84-90.

Forst, S., Delgado, J., Inouye, M., 1989. Phosphorylation of OmpR by the osmosensor EnvZ modulates expression of the OmpF and OmpC genes in *Escherichia coli*. Proceedings of the National Academy of Sciences of the United States of America. 86**,** 6052-6056.

Forster, J., Famili, I., Fu, P., Palsson, B. O., Nielsen, J., 2003. Genome-scale reconstruction of the *Saccharomyces cerevisiae* metabolic network. Genome Research. 13**,** 244-253.

Fortman, J. L., Chhabra, S., Mukhopadhyay, A., Chou, H., Lee, T. S., Steen, E., Keasling, J. D., 2008. Biofuel alternatives to ethanol: pumping the microbial well. Trends in Biotechnology. 26**,** 375-381.

Fox, S. R., Patel, U. A., Yap, M. G. S., Wang, D. I. C., 2004. Maximizing interferon-gamma production by Chinese hamster ovary cells through temperature shift optimization: Experimental and modeling. Biotechnology and Bioengineering. 85**,** 177-184.

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D., Bairoch, A., 2003. ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Research. 31**,** 3784-3788.

GE Healthcare, L. S., Protein Sample Preparation Handbook. 2010.

Genomatica, Genomatica, DuPont Tate & Lyle Bio Products Complete 1st Commercial Production of 1, 4 BDO. Special Chem. Industry News., 2013.

Gestelan.Rf, 1966. Unfolding of *Escherichia coli* ribosomes by removal of magnesium. Journal of Molecular Biology. 18**,** 356-&.

Gevaert, K., Van Damme, P., Ghesquiere, B., Impens, F., Martens, L., Helsens, K., Vandekerckhove, J., 2007. A la carte proteomics with an emphasis on gel-free techniques. Proteomics. 7**,** 2698-2718.

Glick, B. R., Ganoza, M. C., 1976. Characterization and site of action of a soluble protein that stimulates peptide bond synthesis. European Journal of Biochemistry. 71**,** 483-491.

Goldberg, A., 1966. Magnesium binding by *Escherichia coli* ribosomes. Journal of Molecular Biology. 15**,** 663-&.

Gonzalez, R., Murarka, A., Dharmadi, Y., Yazdani, S. S., 2008. A new model for the anaerobic fermentation of glycerol in enteric bacteria: Trunk and auxiliary pathways in *Escherichia coli*. Metabolic Engineering. 10**,** 234-245.

Gonzalez, R., Tao, H., Purvis, J. E., York, S. W., Shanmugam, K. T., Ingram, L. O., 2003. Gene array-based identification of changes that contribute to ethanol tolerance in ethanologenic *Escherichia coli*: Comparison of KO11 (Parent) to LY01 (resistant mutant). Biotechnology Progress. 19**,** 612-623.

Gonzalez, R., Tao, H., Shanmugam, K. T., York, S. W., Ingram, L. O., 2002. Global gene expression differences associated with changes in glycolytic flux and growth rate in *Escherichia coli* during the fermentation of glucose and xylose. Biotechnology Progress. 18**,** 6-20.

Gonzalez, Y. D. a. R., 2009. Elementary network reconstruction: A framework for the analysis of regulatory networks in biological systems. J. Theor. Biol.

Gonzalez-Pajuelo, M., Meynial-Salles, I., Mendes, F., Andrade, J. C., Vasconcelos, I., Soucaille, P., 2005. Metabolic engineering of *Clostridium acetobutylicum* for the industrial production of 1,3-propanediol from glycerol. Metabolic Engineering. 7**,** 329-336.

Gosset, G., 2005. Improvement of *Escherichia coli* production strains by modification of the phosphoenolpyruvate : sugar phosphotransferase system. Microbial Cell Factories. 4.

Graham, R. L., Graham, C., McMullan, G., 2007. Microbial proteomics: a mass spectrometry primer for biologists. Microbial Cell Factories. 6.

Green, J. M., Merkel, W. K., Nichols, B. P., 1992. Characterization and sequence of *Escherichia coli pabC*, the gene encoding aminodeoxychorismate lyase, a pyridoxal phosphate-containing enzyme. Journal of Bacteriology. 174**,** 5317-5323.

Groisman, E. A., 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. Journal of Bacteriology. 183**,** 1835-1842.

Grotkjaer, T., Christakopoulos, P., Nielsen, J., Olsson, L., 2005. Comparative metabolic network analysis of two xylose fermenting recombinant *Saccharomyces cerevisiae* strains. Metabolic Engineering. 7**,** 437-444.

Gupta, A., Murarka, A., Campbell, P., Gonzalez, R., 2009. Anaerobic fermentation of glycerol in *Paenibacillus macerans:* Metabolic pathways and environmental determinants. Applied and Environmental Microbiology. 75**,** 5871-5883.

Gutknecht, R., Beutler, R., Garcia-Alles, L. F., Baumann, U., Erni, B., 2001. The dihydroxyacetone kinase of *Escherichia coli* utilizes a phosphoprotein instead of ATP as phosphoryl donor. Embo Journal. 20**,** 2480-2486.

Halliday, N. M., Hardie, K. R., Williams, P., Winzer, K., Barrett, D. A., 2010. Quantitative liquid chromatography-tandem mass spectrometry profiling of activated methyl cycle metabolites involved in LuxS-dependent quorum sensing in *Escherichia coli.* Analytical Biochemistry. 403**,** 20-29.

Han, M. J., Lee, J. W., Lee, S. Y., Yoo, J. S., 2008. Proteome-level responses of *Escherichia coli* to long-chain fatty acids and use of fatty acid inducible promoter in protein production. Journal of Biomedicine and Biotechnology.

Hanada, K., Kumagai, K., Yasuda, S., Miura, Y., Kawano, M., Fukasawa, M., Nishijima, M., 2003. Molecular machinery for non-vesicular trafficking of ceramide. Nature. 426**,** 803-809.

Harris, L. M., Blank, L., Desai, R. P., Welker, N. E., Papoutsakis, E. T., 2001. Fermentation characterization and flux analysis of recombinant strains of *Clostridium acetobutylicum* with an inactivated *solR* gene. Journal of Industrial Microbiology & Biotechnology. 27**,** 322-328.

Harris, L. M., Desai, R. P., Welker, N. E., Papoutsakis, E. T., 2000. Characterization of recombinant strains of the *Clostridium acetobutylicum* butyrate kinase inactivation mutant: Need for new phenomenological models for solventogenesis and butanol inhibition? Biotechnology and Bioengineering. 67**,** 1-11.

Hassinen, J. B., Durbin, G. T., Bernhart, F. W., 1951. The bacteriostatic effects of saturated fatty acids. Archives of Biochemistry and Biophysics. 31**,** 183-189.

Herring, C. D., Raghunathan, A., Honisch, C., Patel, T., Applebee, M. K., Joyce, A. R., Albert, T. J., Blattner, F. R., van den Boom, D., Cantor, C. R., Palsson, B. O., 2006. Comparative genome sequencing of *Escherichia coli* allows observation of bacterial evolution on a laboratory timescale. Nature Genetics. 38**,** 1406-1412.

Hess, G., 2006. BP and dupont plan 'biobutanol'. Chemical & Engineering News. 84**,** 9-9.

Hieter, P., Boguski, M., 1997. Functional genomics: It's all how you read it. Science. 278**,** 601-602.

Himmi, E. H., Bories, A., Boussaid, A., Hassani, L., 2000. Propionic acid fermentation of glycerol and glucose by *Propionibacterium acidipropionici* and *Propionibacterium freudenreichii* ssp *shermanii*. Applied Microbiology and Biotechnology. 53**,** 435-440.

Hjersted, J. L., Henson, M. A., Mahadevan, R., 2007. Genome-scale analysis of *Saccharomyces cerevisiae* metabolism and ethanol production in fed-batch culture. Biotechnology and Bioengineering. 97**,** 1190-1204.

Hoang, T. T., Sullivan, S. A., Cusick, J. K., Schweizer, H. P., 2002. beta-ketoacyl acyl carrier protein reductase (FabG) activity of the fatty acid biosynthetic pathway is a determining factor of 3-oxo-homoserine lactone acyl chain lengths. Microbiology-Sgm. 148**,** 3849-3856.

Hoenger, A., Pages, J. M., Fourel, D., Engel, A., 1993. The orientation of porin OmpF in the outer membrane of *Escherichia coli*. Journal of Molecular Biology. 233**,** 400-413.

Hoskisson, P. A., Hobbs, G., 2005. Continuous culture - making a comeback? Microbiology-Sgm. 151**,** 3153-3159.

Ideker, T., Galitski, T., Hood, L., 2001a. A new approach to decoding life: Systems biology. Annual Review of Genomics and Human Genetics. 2**,** 343-372.

Ideker, T., Thorsson, V., Ranish, J. A., Christmas, R., Buhler, J., Eng, J. K., Bumgarner, R., Goodlett, D. R., Aebersold, R., Hood, L., 2001b. Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. Science. 292**,** 929-934.

Ishii, N., Nakahigashi, K., Baba, T., Robert, M., Soga, T., Kanai, A., Hirasawa, T., Naba, M., Hirai, K., Hoque, A., Ho, P. Y., Kakazu, Y., Sugawara, K., Igarashi, S., Harada, S., Masuda, T., Sugiyama, N., Togashi, T., Hasegawa, M., Takai, Y., Yugi, K., Arakawa, K., Iwata, N., Toya, Y., Nakayama, Y., Nishioka, T., Shimizu, K., Mori, H., Tomita, M., 2007. Multiple high-throughput analyses monitor the response of *E. coli* to perturbations. Science. 316**,** 593-597.

Ishii, N., Robert, M., Nakayama, Y., Kanai, A., Tomita, M., Toward large-scale modeling of the microbial cell for computer simulation. Vol. 113, 2004, pp. 281-294.

Ito, T., Nakashimada, Y., Senba, K., Matsui, T., Nishio, N., 2005. Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process. Journal of Bioscience and Bioengineering. 100**,** 260-265.

Jafari, S. M., Assadpoor, E., He, Y., Bhandari, B., 2008. Encapsulation efficiency of food flavours and oils during spray drying. Drying Technology. 26, 816-835.

Jantama, K., Haupt, M. J., Svoronos, S. A., Zhang, X. L., Moore, J. C., Shanmugam, K. T., Ingram, L. O., 2008. Combining metabolic engineering and metabolic evolution to develop nonrecombinant strains of *Escherichia coli* C that produce succinate and malate. Biotechnology and Bioengineering. 99, 1140-1153.

Jarboe, L. R., Liu, P., Royce, L. A., 2011. Engineering inhibitor tolerance for the production of biorenewable fuels and chemicals. Current Opinion in Chemical Engineering. 1, 38-42.

Jarboe, L. R., Royce, L. A., Liu, P., 2013. Understanding biocatalyst inhibition by carboxylic acids. Frontiers in Microbiology. 4.

Jayapal, K. R., Wlaschin, K. F., Hu, W.-S., Yap, M. G. S., 2007. Recombinant protein therapeutics from CHO cells - 20 years and counting. Chemical Engineering Progress. 103, 40-47.

Jensen, K. F., Nygaard, P., 1975. Purine nucleoside phosphorylase from *Escherichia coli* and *Salmonella typhimurium*-purification and some properties. European Journal of Biochemistry. 51, 253-265.

Jin, P., Li, S., Lu, S.-g., Zhu, J.-g., Huang, H., 2011. Improved 1,3-propanediol production with hemicellulosic hydrolysates (corn straw) as cosubstrate: Impact of degradation products on *Klebsiella pneumoniae* growth and 1,3-propanediol fermentation. Bioresource Technology. 102, 1815-1821.

Jin, Y. S., Alper, H., Yang, Y. T., Stephanopoulos, G., 2005. Improvement of xylose uptake and ethanol production in recombinant *Saccharomyces cerevisiae* through an inverse metabolic engineering approach. Applied and Environmental Microbiology. 71, 8249-8256.

Joyce, A. R., Palsson, B. O., 2006. The model organism as a system: integrating 'omics' data sets. Nature Reviews Molecular Cell Biology. 7, 198-210.

Kang, Y. S., Durfee, T., Glasner, J. D., Qiu, Y., Frisch, D., Winterberg, K. M., Blattner, F., 2004. Systematic mutagenesis of the *Escherichia coli* genome. Journal of Bacteriology. 186, 4921-4930.

Kantardjieff, A., Jacob, N. M., Yee, J. C., Epstein, E., Kok, Y.-J., Philp, R., Betenbaugh, M., Hu, W.-S., 2010. Transcriptome and proteome analysis of Chinese hamster ovary cells under low temperature and butyrate treatment. Journal of Biotechnology. 145, 143-159.

Karhumaa, K., Pahlman, A. K., Hahn-Hagerdal, B., Levander, F., Gorwa-Grauslund, M. F., 2009. Proteome analysis of the xylose-fermenting mutant yeast strain TMB 3400. Yeast. 26, 371-382.

Kashiwagi, A., Sakurai, T., Tsuru, S., Ying, B. W., Mori, K., Yomo, T., 2009. Construction of *Escherichia coli* gene expression level perturbation collection. Metabolic Engineering. 11, 56-63.

Keasling, J. D., Chou, H., 2008. Metabolic engineering delivers next-generation biofuels. Nature Biotechnology. 26, 298-299.

Kedar, P., Colah, R., Shimizu, K., 2007. Proteomic investigation on the *pykF* gene knockout *Escherichia coli* for aromatic amino acid production. Enzyme and Microbial Technology. 41, 455-465.

Kern, A., Tilley, E., Hunter, I. S., Legisa, M., Glieder, A., 2007. Engineering primary metabolic pathways of industrial micro-organisms. Journal of Biotechnology. 129, 6-29.

Kim, J. Y., Kim, Y.-G., Han, Y. K., Choi, H. S., Kim, Y. H., Lee, G. M., 2011. Proteomic understanding of intracellular responses of recombinant Chinese hamster ovary cells cultivated in serum-free medium supplemented with hydrolysates. Applied Microbiology and Biotechnology. 89, 1917-1928.

Kim, J. Y., Kim, Y.-G., Lee, G. M., 2012. Differential in-gel electrophoresis (DIGE) analysis of CHO cells under hyperosmotic pressure: Osmoprotective effect of glycine betaine addition. Biotechnology and Bioengineering. 109, 1395-1403.

Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E., Toyonaga, H., Mori, H., 2005. Complete set of ORF clones of *Escherichia coli* ASKA library (A complete Set of *E. coli* K-12 ORF archive): Unique resources for biological research. DNA Research. 12, 291-299.

Kizer, L., Pitera, D. J., Pfleger, B. F., Keasling, J. D., 2008. Application of functional genomics to pathway optimization for increased isoprenoid production. Applied and Environmental Microbiology. 74, 3229-3241.

Kocabaş, P., Çalik, P., Çalik, G., Özdamar, T., 2009. Microarray studies in *Bacillus subtilis.* Biotechnology Journal. 4, 1012-1027.

Koffas, M., Stephanopoulos, G., 2005. Strain improvement by metabolic engineering: lysine production as a case study for systems biology. Current Opinion in Biotechnology. 16, 361-366.

Koide, T., Pang, W., Baliga, N., 2009. The role of predictive modelling in rationally re-engineering biological systems. Nature Rev. Microbiology. 7, 297-305.

Kolkman, A., Olsthoorn, M. M. A., Heeremans, C. E. M., Heck, A. J. R., Slijper, M., 2005. Comparative proteome analysis of *Saccharomyces cerevisiae* grown in chemostat cultures limited for glucose or ethanol. Molecular & Cellular Proteomics. 4, 1-11.

Korteland, J., Tommassen, J., Lugtenberg, B., 1982. PhoE protein pore of the outer membrane of *Escherichia coli* K-12 is a particularly efficient channel for organic and inorganic phosphate. Biochimica Et Biophysica Acta. 690, 282-289.

Kou, T.-C., Fan, L., Zhou, Y., Ye, Z.-Y., Liu, X.-P., Zhao, L., Tan, W.-S., 2011. Detailed understanding of enhanced specific productivity in Chinese hamster ovary cells at low culture temperature. Journal of Bioscience and Bioengineering. 111, 365-369.

Kretzmer, G., 2002. Industrial processes with animal cells. Applied Microbiology and Biotechnology. 59, 135-142.

Krishnan, K., Flower, A. M., 2008. Suppression of delta *bipA* phenotypes in *Escherichia coli* by abolishment of pseudouridylation at specific sites on the 23S rRNA. Journal of Bacteriology. 190, 7675-7683.

Kuystermans, D., Krampe, B., Swiderek, H., Al-Rubeai, M., 2007. Using cell engineering and omic tools for the improvement of cell culture processes. Cytotechnology. 53, 3-22.

Ladygina, N., Dedyukhina, E. G., Vainshtein, M. B., 2006. A review on microbial synthesis of hydrocarbons. Process Biochemistry. 41, 1001-1014.

Leblanc, G., Rimon, G., Kaback, H. R., 1980. Glucose-6-phosphate transport in membrane vesicles isolated from *Escherichia coli* - Effect of imposed electrical potential and pH gradient. Biochemistry. 19, 2522-2528.

Lee, J., Yun, H., Feist, A. M., Palsson, B. O., Lee, S. Y., 2008a. Genome-scale reconstruction and *in silico* analysis of the *Clostridium acetobutylicum* ATCC 824 metabolic network. Applied Microbiology and Biotechnology. 80, 849-862.

Lee, K. H., Park, J. H., Kim, T. Y., Kim, H. U., Lee, S. Y., 2007. Systems metabolic engineering of *Escherichia coli* for L-threonine production. Molecular Systems Biology. 3.

Lee, P. C., Lee, W. G., Lee, S. Y., Chang, H. N., 2001. Succinic acid production with reduced by-product formation in the fermentation of *Anaerobiospirillum succiniciproducens* using glycerol as a carbon source. Biotechnology and Bioengineering. 72, 41-48.

Lee, S. K., Chou, H., Ham, T. S., Lee, T. S., Keasling, J. D., 2008b. Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels. Current Opinion in Biotechnology. 19, 556-563.

Lee, S. Y., Hong, S. H., Lee, S. H., Park, S. J., 2004. Fermentative production of chemicals that can be used for polymer synthesis. Macromolecular Bioscience. 4**,** 157-164.

Lee, S. Y., Lee, D. Y., Kim, T. Y., 2005. Systems biotechnology for strain improvement. Trends in Biotechnology. 23**,** 349-358.

Legras, J. L., Erny, C., Le Jeune, C., Lollier, M., Adolphe, Y., Demuyter, C., Delobel, P., Blondin, B., Karst, F., 2010. Activation of two different resistance mechanisms in *Saccharomyces cerevisiae* upon exposure to octanoic and decanoic acids. Appl. Environ. Microbiol. 76**,** 7526-7535.

Lehti, T. A., Bauchart, P., Dobrindt, U., Korhonen, T. K., Westerlund-Wikstrom, B., 2012. The fimbriae activator MatA switches off motility in *Escherichia coli* by repression of the flagellar master operon *flhDC*. Microbiology (Reading, England). 158**,** 1444-55.

Lenahan, M., Sheridan, A., Morris, D., Duffy, G., Fanning, S., Burgess, C. M., 2014. Transcriptomic analysis of triclosan-susceptible and -tolerant *Escherichia coli* O157: H19 in response to triclosan exposure. Microbial Drug Resistance. 20**,** 91-103.

Lennen, R. M., Kruziki, M. A., Kumar, K., Zinkel, R. A., Burnum, K. E., Lipton, M. S., Hoover, S. W., Ranatunga, D. R., Wittkopp, T. M., Marner, W. D., II, Pfleger, B. F., 2011. Membrane stresses induced by overproduction of free fatty acids in *Escherichia coli.* Appl. Environ. Microbiol. 77**,** 8114-8128.

Lennen, R. M., Pfleger, B. F., 2013. Modulating Membrane Composition Alters Free Fatty Acid Tolerance in *Escherichia coli*. Plos One. 8.

Lennen, R. M., Politz, M. G., Kruziki, M. A., Pfleger, B. F., 2013. Identification of Transport Proteins Involved in Free Fatty Acid Efflux in *Escherichia coli.* Journal of Bacteriology. 195**,** 135-144.

Li, K. Y., Pasternak, C., Klug, G., 2003. Expression of the *trxA* gene for thioredoxin 1 in *Rhodobacter sphaeroides* during oxidative stress. Archives of Microbiology. 180**,** 484-489.

Li, M., Zhang, X., Agrawal, A., San, K.-Y., 2012. Effect of acetate formation pathway and long chain fatty acid CoA-ligase on the free fatty acid production in *E. coli* expressing acy-ACP thioesterase from *Ricinus communis*. Metabolic Engineering. 14**,** 380-387.

Liao, J. C., Higashide, W., 2008. Metabolic engineering of next-generation biofuels. Chemical Engineering Progress. 104**,** S19-S23.

Lim, Y., Wong, N. S. C., Lee, Y. Y., Ku, S. C. Y., Wong, D. C. F., Yap, M. G. S., 2010. Engineering mammalian cells in bioprocessing - current achievements and future perspectives. Biotechnology and Applied Biochemistry. 55**,** 175-189.

Lin, E. C. C., 1976. Glycerol dissimilation and its regulation in bacteria. Annual Review of Microbiology. 30**,** 535-578.

Lin, F. M., Qiao, B., Yuan, Y. J., 2009. Comparative Proteomic Analysis of Tolerance and Adaptation of Ethanologenic *Saccharomyces cerevisiae* to Furfural, a Lignocellulosic Inhibitory Compound. Applied and Environmental Microbiology. 75**,** 3765-3776.

Lin, X., Wang, C., Guo, C., Tian, Y., Li, H., Peng, X., 2012. Differential regulation of OmpC and OmpF by AtpB in *Escherichia coli* exposed to nalidixic acid and chlortetracycline. Journal of Proteomics. 75**,** 5898-5910.

Liolios, K., Mavromatis, K., Tavernarakis, N., Kyrpides, N. C., 2008. The Genomes On Line Database (GOLD) in 2007: status of genomic and metagenomic projects and their associated metadata. Nucleic Acids Research. 36**,** D475-D479.

Liscum, L., Munn, N. J., 1999. Intracellular cholesterol transport. Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids. 1438**,** 19-37.

Liu, H., Yu, C., Feng, D., Cheng, T., Meng, X., Liu, W., Zou, H., Xian, M., 2012. Production of extracellular fatty acid using engineered *Escherichia coli.* Microbial Cell Factories. 11.

Liu, J., Weng, Z., Wang, Y., Chao, H., Zongwan, M., 2009. Metabolic engineering based on systems biology for chemicals production. Front. Biol. China. 4**,** 260-265.

Liu, P., Chernyshov, A., Najdi, T., Fu, Y., Dickerson, J., Sandmeyer, S., Jarboe, L., 2013. Membrane stress caused by octanoic acid in *Saccharomyces cerevisiae*. Applied Microbiology and Biotechnology. 97**,** 3239-3251.

Liu, X. Q., Ferenci, T., 1998. Regulation of porin-mediated outer membrane permeability by nutrient limitation in *Escherichia coli*. Journal of Bacteriology. 180**,** 3917-3922.

Lloyd, C. M., Lawson, J. R., Hunter, P. J., Nielsen, P. F., 2008. The CellML model repository. Bioinformatics. 24**,** 2122-2123.

Lockhart, D. J., Winzeler, E. A., 2000. Genomics, gene expression and DNA arrays. Nature. 405**,** 827-836.

Lodmell, J. S., Dahlberg, A. E., 1997. A conformational switch in *Escherichia coli* 16S ribosomal RNA during decoding of messenger RNA. Science. 277**,** 1262-1267.

Lu, X., Vora, H., Khosla, C., 2008. Overproduction of free fatty acids in *E. coli*: Implications for biodiesel production. Metabolic Engineering. 10**,** 333-339.

Lubke, C., Boidol, W., Petri, T., 1995. Analysis and optimization of recombinant protein production in *Escherichia coli* using the inducible Pho-A promoter of the *Escherichia coli* alkaline-phosphatase. Enzyme and Microbial Technology. 17**,** 923-928.

Lugtenberg, B., 1981. Composition and function of the outer membran of *Escherichia coli*. Trends in Biochemical Sciences. 6**,** 262-266.

MacLean, M. J., Ness, L. S., Ferguson, G. P., Booth, I. R., 1998. The role of glyoxalase I in the detoxification of methylglyoxal and in the activation of the KefB K^+ efflux system in *Escherichia coli*. Molecular Microbiology. 27**,** 563-571.

Marchant, R. J., Al-Fageeh, M. B., Underhill, M. F., Racher, A. J., Smales, C. M., 2008. Metabolic rates, growth phase, and mRNA levels influence cell-specific antibody production levels from in vitro-cultured mammalian cells at sub-physiological temperatures. Molecular Biotechnology. 39**,** 69-77.

Marounek, M., Skrivanova, E., Rada, V., 2003. Susceptibility of *Escherichia coli* to C-2-C-18 fatty acids. Folia Microbiologica. 48**,** 731-735.

Martin, V. J. J., Pitera, D. J., Withers, S. T., Newman, J. D., Keasling, J. D., 2003. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nature Biotechnology. 21**,** 796-802.

Mashego, M. R., Jansen, M. L. A., Vinke, J. L., van Gulik, W. M., Heijnen, J. J., 2005. Changes in the metabolome of *Saccharomyces cerevisiae* associated with evolution in aerobic glucose-limited chemostats. Fems Yeast Research. 5**,** 419-430.

Matsushika, A., Inoue, H., Kodaki, T., Sawayama, S., 2009. Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: current state and perspectives. Applied Microbiology and Biotechnology. 84**,** 37-53.

Mattam, A. J., Clomburg, J. M., Gonzalez, R., Yazdani, S. S., 2013. Fermentation of glycerol and production of valuable chemical and biofuel molecules. Biotechnology Letters. 35**,** 831-842.

Maurer, L. M., Yohannes, E., Bondurant, S. S., Radmacher, M., Slonczewski, J. L., 2005. pH regulates genes for flagellar motility, catabolism, and oxidative stress in *Escherichia coli* K-12. Journal of Bacteriology. 187**,** 304-319.

Mazumdar, S., Blankschien, M. D., Clomburg, J. M., Gonzalez, R., 2013. Efficient synthesis of L-lactic acid from glycerol by metabolically engineered *Escherichia coli*. Microbial Cell Factories. 12.

Mazumdar, S., Clomburg, J. M., Gonzalez, R., 2010. *Escherichia coli* Strains Engineered for Homofermentative Production of D-Lactic Acid from Glycerol. Applied and Environmental Microbiology. 76**,** 4327-4336.

McCord, J. M., Fridovic.I, 1969. Superoxide dismutase- an enzymic function for erythrocuprein (hemocuprein). Journal of Biological Chemistry. 244**,** 6049-&.

McKendry, P., 2002. Energy production from biomass (part 2): conversion technologies. Bioresource Technology. 83**,** 47-54.

Mehta, B., Kathalewar, M., Sabnis, A., 2014. Diester Based on Castor Oil Fatty Acid as Plasticizer for Poly(vinyl chloride). Journal of Applied Polymer Science. 131**,** 8.

Metsoviti, M., Paramithiotis, S., Drosinos, E. H., Galiotou-Panayotou, M., Nychas, G.-J. E., Zeng, A.-P., Papanikolaou, S., 2012. Screening of bacterial strains capable of converting biodiesel-derived raw glycerol into 1,3-propanediol, 2,3-butanediol and ethanol. Engineering in Life Sciences. 12**,** 57-68.

Meyer, E., Leonard, N. J., Bhat, B., Stubbe, J., Smith, J. M., 1992. Purification and characterization of the *purE, purK*, and *purC* gene products- Identification of a previously unrecognized energy requirement in the purine biosynthetic pathway.. Biochemistry. 31**,** 5022-5032.

Meyer, S. E., Granett, S., Jung, J. U., Villarejo, M. R., 1990. Osmotic regulation of PhoE porin synthesis in *Escherichia coli*. Journal of Bacteriology. 172**,** 5501-5502.

Michalak, M., Corbett, E. F., Mesaeli, N., Nakamura, K., Opas, M., 1999. Calreticulin: one protein, one gene, many functions. Biochemical Journal. 344**,** 281-292.

Miedema, H., Vrouenraets, M., Wierenga, J., Gillespie, D., Eisenberg, B., Meijberg, W., Nonner, W., 2006. Ca^{2+} selectivity of a chemically modified OmpF with reduced pore volume. Biophysical Journal. 91**,** 4392-4400.

Miller, E. N., Jarboe, L. R., Turner, P. C., Pharkya, P., Yomano, L. P., York, S. W., Nunn, D., Shanmugam, K. T., Ingram, L. O., 2009a. Furfural Inhibits Growth by Limiting Sulfur Assimilation in Ethanologenic *Escherichia coli* Strain LY180. Applied and Environmental Microbiology. 75**,** 6132-6141.

Miller, E. N., Jarboe, L. R., Yomano, L. P., York, S. W., Shanmugam, K. T., Ingram, L. O., 2009b. Silencing of NADPH-Dependent Oxidoreductase Genes (*yqhD* and *dkgA*) in Furfural-Resistant Ethanologenic *Escherichia coli*. Applied and Environmental Microbiology. 75**,** 4315-4323.

Miller, J. H., 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Molloy, M. P., Herbert, B. R., Slade, M. B., Rabilloud, T., Nouwens, A. S., Williams, K. L., Gooley, A. A., 2000. Proteomic analysis of the *Escherichia coli* outer membrane. European Journal of Biochemistry. 267**,** 2871-2881.

Moon, C., Lee, C. H., Sang, B.-I., Um, Y., 2011. Optimization of medium compositions favoring butanol and 1,3-propanediol production from glycerol by *Clostridium pasteurianum*. Bioresource Technology. 102**,** 10561-10568.

Mueller, W. E. G., Wang, X., Grebenjuk, V. A., Korzhev, M., Wiens, M., Schlossmacher, U., Schroeder, H. C., 2012. Common Genetic Denominators for Ca^{++}-Based Skeleton in Metazoa: Role of Osteoclast-Stimulating Factor and of Carbonic Anhydrase in a Calcareous Sponge. Plos One. 7.

Mukhopadhyay, A., Redding, A. M., Rutherford, B. J., Keasling, J. D., 2008. Importance of systems biology in engineering microbes for biofuel production. Current Opinion in Biotechnology. 19**,** 228-234.

Muller, D. J., Engel, A., 1999. Voltage and pH-induced channel closure of porin OmpF visualized by atomic force microscopy. Journal of Molecular Biology. 285**,** 1347-1351.

Murarka, A., Dharmadi, Y., Yazdani, S. S., Gonzalez, R., 2008. Fermentative utilization of glycerol by *Escherichia coli* and its implications for the production of fuels and chemicals. Applied and Environmental Microbiology. 74**,** 1124-1135.

Nakae, T., 1976. Identification of outer membrane protein of *Escherichia coli* that produces transmembrane channels in reconstituted vesicle membranes .11. Biochemical and Biophysical Research Communications. 71**,** 877-884.

Nakamura, H., Hase, A., 1984. Effect of lauric acid on cell division, macromolecular synthesis and membrane lipid organization in *Escherichia coli.* Plant and Cell Physiology. 25**,** 167-175.

Nakanishi, N., Tashiro, K., Kuhara, S., Hayashi, T., Sugimoto, N., Tobe, T., 2009. Regulation of virulence by butyrate sensing in enterohaemorrhagic *Escherichia coli.* Microbiology-Sgm. 155**,** 521-530.

Neidhard.Fc, Bloch, P. L., Smith, D. F., 1974. Culture medium for enterobacteria. Journal of Bacteriology. 119**,** 736-747.

Nielsen, J., Villadsen, J., Liden, G., 2003. Bioreaction Engineering Principles. Kluwer Academic/Plenum Publishers, New York.

Nikolau, B. J., Perera, M., Brachova, L., Shanks, B., 2008. Platform biochemicals for a biorenewable chemical industry. Plant Journal. 54**,** 536-545.

Nunn, W. D., Colburn, R. W., Black, P. N., 1986. Transport of long-chain fatty acids in *Escherichia coli*- Evidence for role of *fadL* gene product as long-chain fatty acid receptor. Journal of Biological Chemistry. 261**,** 167-171.

Ofarrell, P. H., 1975. High resolution 2-dimensional electrophoresis of proteins. Journal of Biological Chemistry. 250**,** 4007-4021.

Oh, B.-R., Seo, J.-W., Heo, S.-Y., Hong, W.-K., Luo, L. H., Joe, M.-h., Park, D.-H., Kim, C. H., 2011. Efficient production of ethanol from crude glycerol by a *Klebsiella pneumoniae* mutant strain. Bioresource Technology. 102**,** 3918-3922.

Oren, A., 2005. A hundred years of *Dunaliella* research: 1905-2005. Saline systems. 1**,** 2-2.

Otero, J. M., Panagiotou, G., Olsson, L., 2007. Fueling industrial biotechnology growth with bioethanol. Biofuels. vol. 108. Springer-Verlag Berlin, Berlin, pp. 1-40.

Paredes, C. J., Alsaker, K. V., Papoutsakis, E. T., 2005. A comparative genomic view of clostridial sporulation and physiology. Nature Reviews Microbiology. 3**,** 969-978.

Park, J. H., Lee, K. H., Kim, T. Y., Lee, S. Y., 2007. Metabolic engineering of *Escherichia coli* for the production of L-valine based on transcriptome analysis and *in silico* gene knockout simulation. Proceedings of the National Academy of Sciences of the United States of America. 104**,** 7797-7802.

Park, J. H., Lee, S. Y., 2008. Towards systems metabolic engineering of microorganisms for amino acid production. Current Opinion in Biotechnology. 19**,** 454-460.

Park, M. S., Hill, C. M., Li, Y. C., Hardy, R. K., Khanna, H., Khang, Y. H., Raushel, F. M., 2004. Catalytic properties of the PepQ prolidase from *Escherichia coli.* Archives of Biochemistry and Biophysics. 429**,** 224-230.

Paulsen, I. T., Reizer, J., Jin, R. Z., Lin, E. C. C., Saier, M. H., 2000. Functional genomic studies of dihydroxyacetone utilization in *Escherichia coli.* Microbiology-Uk. 146**,** 2343-2344.

Pavlou, A. K., Reichert, J. M., 2004. Recombinant protein therapeutics - success rates, market trends and values to 2010. Nature Biotechnology. 22**,** 1513-1519.

Peng, L. F., Shimizu, K., 2006. Effect of *fadR* gene knockout on the metabolism of *Escherichia coli* based on analyses of protein expressions, enzyme activities and intracellular metabolite concentrations. Enzyme and Microbial Technology. 38**,** 512-520.

Piette, J., Nyunoya, H., Lusty, C. J., Cunin, R., Weyens, G., Crabeel, M., Charlier, D., Glansdorff, N., Pierard, A., 1984. DNA-sequence of the *carA* gene and the control region of *carAB*- Tandem promoters, respectively controlled by arginine and the pyrimidines, regulate the synthesis of carbamoyl-phosphate synthetase in *Escherichia coli* K-12. Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences. 81**,** 4134-4138.

Pitkanen, J. P., Aristidou, A., Salusjarvi, L., Ruohonen, L., Penttila, M., 2003. Metabolic flux analysis of xylose metabolism in recombinant *Saccharomyces cerevisiae* using continuous culture. Metabolic Engineering. 5**,** 16-31.

Postma, P. W., Lengeler, J. W., Jacobson, G. R., 1993. Phosphoenolpyruvate-carbohydrate phosphotransferase systems of bacteria. Microbiological Reviews. 57**,** 543-594.

Pozzan, T., Rizzuto, R., Volpe, P., Meldolesi, J., 1994. Molecular and cellular physiology of intracellular calcium stores. Physiological Reviews. 74**,** 595-636.

Prajapati, H. N., Dalrymple, D. M., Serajuddin, A. T. M., 2012. A Comparative Evaluation of Mono-, Di- and Triglyceride of Medium Chain Fatty Acids by Lipid/Surfactant/Water Phase Diagram, Solubility Determination and Dispersion Testing for Application in Pharmaceutical Dosage Form Development. Pharmaceutical Research. 29**,** 285-305.

Price, N. D., Papin, J. A., Schilling, C. H., Palsson, B. O., 2003. Genome-scale microbial *in silico* models: the constraints-based approach. Trends in Biotechnology. 21**,** 162-169.

Quastel, J. H., Stephenson, M., 1925. Further observations on the anaerobic growth of bacteria. Biochemical Journal. 19**,** 660-666.

Quastel, J. H., Stephenson, M., Whetham, M. D., 1925. Some reactions of resting bacteria in relation to anaerobic growth. Biochemical Journal. 19**,** 304-317.

Queralt Martin, M., Alcaraz, A., Aguilella, V. M., 2013. Current Fluctuation Analysis to Study Mg^{2+}-Binding in the Bacterial Porin OmpF. Biophysical Journal. 104**,** 630A-630A.

Raman, K., Chandra, N., 2009. Flux balance analysis of biological systems: applications and challenges. Briefings in Bioinformatics. 10**,** 435-449.

Rath, B. B., 2009. Harvesting Alternate Energies from Our Planet. JOM Journal of the Minerals, Metals and Materials Society. 61**,** 73-78.

Reed, J. L., Vo, T. D., Schilling, C. H., Palsson, B. O., 2003. An expanded genome-scale model of *Escherichia coli* K-12 (iJR904 GSM/GPR). Genome Biology. 4.

Reinhart, R. A., 1988. Magnesium metabolism - A review with special reference to the relationship between intracellular content and serum levels. Archives of Internal Medicine. 148**,** 2415-2420.

Ricke, S. C., 2003. Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. Poultry Science. 82**,** 632-639.

Ried, G., Hindennach, I., Henning, U., 1990. Role of the lipopolysaccharide in assembly of *Escherichia coli* outer membrane proteins OmpA, OmpC, and OmpF. Journal of Bacteriology. 172**,** 6048-6053.

Rizzi, M., Baltes, M., Theobald, U., Reuss, M., 1997. *In vivo* analysis of metabolic dynamics in *Saccharomyces cerevisiae* .2. Mathematical model. Biotechnology and Bioengineering. 55**,** 592-608.

Ro, S., An annotated history of oil prices since 1861. Goldman Sachs, 2014.

Robertson, K. M., Tieleman, D. P., 2002. Molecular basis of voltage gating of OmpF porin. Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire. 80**,** 517-523.

Rodriguez-Moya, M. a. G., R, 2010. Systems biology approaches for the microbial production of biofuels. Biofuels. 1**,** 291-310.

Rollins, D. K., Zhai, D. M., Joe, A. L., Guidarelli, J. W., Murarka, A., Gonzalez, R., 2006. A novel data mining method to identify assay-specific signatures in functional genomic studies. Bmc Bioinformatics. 7.

Roodman, G. D., 1996. Advances in bone biology: The osteoclast. Endocrine Reviews. 17**,** 308-332.

Rose, I. A., Nowick, J. S., 2002. Methylglyoxal synthetase, enol-pyruvaidehyde, glutathione and the glyoxalase system. Journal of the American Chemical Society. 124**,** 13047-13052.

Royce, L. A., Boggess, E., Fu, Y., Liu, P., Shanks, J. V., Dickerson, J., Jarboe, L. R., 2014. Transcriptomic Analysis of Carboxylic Acid Challenge in *Escherichia coli*: Beyond Membrane Damage. Plos One. 9.

Royce, L. A., Liu, P., Stebbins, M. J., Hanson, B. C., Jarboe, L. R., 2013. The damaging effects of short chain fatty acids on *Escherichia coli* membranes. Applied Microbiology and Biotechnology. 97**,** 8317-8327.

Saffen, D. W., Presper, K. A., Doering, T. L., Roseman, S., 1987. Sugar transport by the bacterial phosphotransferase system. 30.Molecular cloning ad structural analysis of the Escherichia coli *ptsH, ptsI,* and *crr* genes. Journal of Biological Chemistry. 262**,** 16241-16253.

Sahm, H., Eggeling, L., Eikmanns, B., Kramer, R., 1996. Construction of L-lysine-, L-threonine-, or L-isoleucine-overproducing strains of *Corynebacterium glutamicum*. Recombinant DNA Biotechnology Iii: the Integration of Biological and Engineering Sciences. 782**,** 25-39.

Salem, A. R., Foster, M. A., 1972. Microbial biosynthesis of methionine. Biochemical Journal. 127**,** 845-&.

Salusjarvi, L., Kankainen, M., Soliymani, R., Pitkanen, J. P., Penttila, M., Ruohonen, L., 2008. Regulation of xylose metabolism in recombinant *Saccharomyces cerevisiae*. Microbial Cell Factories. 7.

Salusjarvi, L., Pitkanen, J. P., Aristidou, A., Ruohonen, L., Penttila, M., 2006. Transcription analysis of recombinant *Saccharomyces cerevisiae* reveals novel responses to xylose. Applied Biochemistry and Biotechnology. 128**,** 237-261.

Sambrook, J., Russell, D., 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY.

Sauer, U., 2001. Evolutionary engineering of industrially important microbial phenotypes. Advances in Biochemical Engineering/Biotechnology. 73**,** 129-169.

Sauer, U., Lasko, D. R., Fiaux, J., Hochuli, M., Glaser, R., Szyperski, T., Wuthrich, K., Bailey, J. E., 1999. Metabolic flux ratio analysis of genetic and environmental modulations of *Escherichia coli* central carbon metabolism. Journal of Bacteriology. 181**,** 6679-6688.

Schaffer, S., Isci, N., Zickner, B., Durre, P., 2002. Changes in protein synthesis and identification of proteins specifically induced during solventogenesis in *Clostridium acetobutylicum*. Electrophoresis. 23**,** 110-121.

Schatz, S. M., Kerschbaumer, R. J., Gerstenbauer, G., Kral, M., Dorner, F., Scheiflinger, F., 2003. Higher expression of Fab antibody fragments in a CHU cell line at reduced temperature. Biotechnology and Bioengineering. 84**,** 433-438.

Schellhorn, H. E., Hassan, H. M., 1988. Response of hydroperoxidase and superoxide dismutase-deficient mutants of *Escherichia coli* K-12 to oxidative stress. Canadian Journal of Microbiology. 34**,** 1171-1176.

Schenkman, J. B., Jansson, I., 2003. The many roles of cytochrome b(5). Pharmacology & Therapeutics. 97**,** 139-152.

Schirch, V., Hopkins, S., Villar, E., Angelaccio, S., 1985. Serine hydroxymethyltransferase from *Escherichia coli*- Purification and properties. Journal of Bacteriology. 163**,** 1-7.

Schmeisser, C., Steele, H., Streit, W. R., 2007. Metagenomics, biotechnology with non-culturable microbes. Applied Microbiology and Biotechnology. 75**,** 955-962.

Scholten, E., Daegele, D., 2008. Succinic acid production by a newly isolated bacterium. Biotechnology Letters. 30**,** 2143-2146.

Schonbrunner, E. R., Schmid, F. X., 1992. Peptidyl-prolyl *cis-trans* isomerase improves the efficiency of protein disulfide isomerase as a catalyst of protein folding. Proceedings of the National Academy of Sciences of the United States of America. 89**,** 4510-4513.

Schulz, G. E., 2002. The structure of bacterial outer membrane proteins. Biochimica Et Biophysica Acta-Biomembranes. 1565, 308-317.

Segura, A., Molina, L., Fillet, S., Krell, T., Bernal, P., Munoz-Rojas, J., Ramos, J.-L., 2012. Solvent tolerance in Gram-negative bacteria. Curr. Opin. Biotechnol. 23, 415-421.

Selvarasu, S., Ho, Y. S., Chong, W. P. K., Wong, N. S. C., Yusufi, F. N. K., Lee, Y. Y., Yap, M. G. S., Lee, D.-Y., 2012. Combined *in silico* modeling and metabolomics analysis to characterize fed-batch CHO cell culture. Biotechnology and Bioengineering. 109, 1415-1429.

Senger, R. S., Papoutsakis, E. T., 2008a. Genome-Scale Model for *Clostridium acetobutylicum*: Part I. Metabolic Network Resolution and Analysis. Biotechnology and Bioengineering. 101, 1036-1052.

Senger, R. S., Papoutsakis, E. T., 2008b. Genome-Scale Model for *Clostridium acetobutylicum*: Part II. Development of Specific Proton Flux States and Numerically Determined Sub-Systems. Biotechnology and Bioengineering. 101, 1053-1071.

Seo, J. S., Chong, H. Y., Park, H. S., Yoon, K. O., Jung, C., Kim, J. J., Hong, J. H., Kim, H., Kim, J. H., Kil, J. I., Park, C. J., Oh, H. M., Lee, J. S., Jin, S. J., Um, H. W., Lee, H. J., Oh, S. J., Kim, J. Y., Kang, H. L., Lee, S. Y., Lee, K. J., Kang, H. S., 2005. The genome sequence of the ethanologenic bacterium *Zymomonas mobilis* ZM4. Nature Biotechnology. 23, 63-68.

Serres, M. H., Gopal, S., Nahum, L. A., Liang, P., Gaasterland, T., Riley, M., 2001. A functional update of the *Escherichia coli* K-12 genome. Genome Biology. 2.

Seth, G., Charaniya, S., Wiaschin, K. F., Hu, W.-S., 2007a. In pursuit of a super producer - alternative paths to high producing recombinant mammalian cells. Current Opinion in Biotechnology. 18, 557-564.

Seth, G., Philp, R. J., Lau, A., Jiun, K. Y., Yap, M., Hu, W.-S., 2007b. Molecular portrait of high productivity in recombinant NS0 cells. Biotechnology and Bioengineering. 97, 933-951.

Shen, C. R., Liao, J. C., 2008. Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. Metabolic Engineering. 10, 312-320.

Shen, D., Kiehl, T. R., Khattak, S. F., Li, Z. J., He, A., Kayne, P. S., Patel, V., Neuhaus, I. M., Sharfstein, S. T., 2010. Transcriptomic Responses to Sodium Chloride-Induced Osmotic Stress: A Study of Industrial Fed-Batch CHO Cell Cultures. Biotechnology Progress. 26, 1104-1115.

Sheridan, A., Lenahan, M., Condell, O., Bonilla-Santiago, R., Sergeant, K., Renaut, J., Duffy, G., Fanning, S., Nally, J. E., Burgess, C. M., 2013. Proteomic and phenotypic analysis of triclosan tolerant verocytotoxigenic *Escherichia coli* O157:H19. Journal of Proteomics. 80, 78-90.

Shi, D. J., Wang, C. L., Wang, K. M., 2009a. Genome shuffling to improve thermotolerance, ethanol tolerance and ethanol productivity of *Saccharomyces cerevisiae*. Journal of Industrial Microbiology & Biotechnology. 36, 139-147.

Shi, S., Chen, T., Zhang, Z., Chen, X., Zhao, X., 2009b. Transcriptome analysis guided metabolic engineering of *Bacillus subtilis* for riboflavin production. Metabolic Engineering. 11, 243-252.

Shi, Z., Blaschek, H. P., 2008. Transcriptional Analysis of *Clostridium beijerinckii* NCIMB 8052 and the Hyper-Butanol-Producing Mutant BA101 during the Shift from Acidogenesis to Solventogenesis. Applied and Environmental Microbiology. 74, 7709-7714.

Shilling, O., Frick, O., Herzberg, C., Ehrenreich, A., Heinzle, E., Wittmann, C., Stülke, J., 2007. Transcriptional and Metabolic Responses of *Bacillus subtilis* to the Availability of Organic Acids: Transcription Regulation is Important but Not Sufficient to Account for Metabolic Adaptation. Applied and Environmental Microbiology. 73, 499-507.

Shinagawa, H., Makino, K., Nakata, A., 1983. Regulation of the *pho* regulon in *Escherichia coli* K-12- Genetic and physiological regulation of the positive regulatory gene *phoB*. Journal of Molecular Biology. 168**,** 477-488.

Simpson, T. W., Shimizu, H., Stephanopoulos, G., 1998. Experimental determination of group flux control coefficients in metabolic networks. Biotechnology and Bioengineering. 58**,** 149-153.

Sirko, A., Weglenska, A., Hryniewicz, M., Hulanicka, D. M., 1997. Characterization of the *Escherichia coli* gene encoding a new member of the short-chain dehydrogenase/reductase (SDR) family. Acta Biochimica Polonica. 44**,** 153-157.

Smith, A. M., 2008. Prospects for increasing starch and sucrose yields for bioethanol production. Plant Journal. 54**,** 546-558.

Smith, D. R., Quinlan, A. R., Peckham, H. E., Makowsky, K., Tao, W., Woolf, B., Shen, L., Donahue, W. F., Tusneem, N., Stromberg, M. P., Stewart, D. A., Zhang, L., Ranade, S. S., Warner, J. B., Lee, C. C., Coleman, B. E., Zhang, Z., McLaughlin, S. F., Malek, J. A., Sorenson, J. M., Blanchard, A. P., Chapman, J., Hillman, D., Chen, F., Rokhsar, D. S., McKernan, K. J., Jeffries, T. W., Marth, G. T., Richardson, P. M., 2008. Rapid whole-genome mutational profiling using next-generation sequencing technologies. Genome Research. 18**,** 1638-1642.

Snoep, J. L., Olivier, B. G., 2003. JWS online cellular systems modelling and microbiology. Microbiology-Sgm. 149**,** 3045-3047.

Soltys, B. J., Gupta, R. S., 1997. Cell surface localization of the 60 kDa heat shock chaperonin protein (hsp60) in mammalian cells. Cell Biology International. 21**,** 315-320.

Sommer, B., Friehs, K., Flaschel, E., Reck, M., Stahl, F., Scheper, T., 2009. Extracellular production and affinity purification of recombinant proteins with *Escherichia coli* using the versatility of the maltose binding protein. Journal of Biotechnology. 140**,** 194-202.

Spring, T. G., Wold, F., 1971. Purification and characterization of *Escherichia coli* enolase. Journal of Biological Chemistry. 246**,** 6797-&.

Sprong, R. C., Hulstein, M. F. E., Van der Meer, R., 2001. Bactericidal activities of milk lipids. Antimicrobial Agents and Chemotherapy. 45**,** 1298-1301.

Steen, E. J., Kang, Y. S., Bokinsky, G., Hu, Z. H., Schirmer, A., McClure, A., del Cardayre, S. B., Keasling, J. D., 2010. Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. Nature. 463**,** 559-U182.

Stephanopoulos, G., 2007. Challenges in engineering microbes for biofuels production. Science. 315**,** 801-804.

Stephanopoulos, G., Alper, H., Moxley, J., 2004. Exploiting biological complexity for strain improvement through systems biology. Nature Biotechnology. 22**,** 1261-1267.

Stephanopoulos, G., Jin, Y. S., Alper, H., Inverse metabolic engineering. Vol. 118, 2005, pp. S93-S93.

Stephanopoulos, G., Stafford, D. E., 2002. Metabolic engineering: a new frontier of chemical reaction engineering. Chemical Engineering Science. 57**,** 2595-2602.

Stock, A. M., Robinson, V. L., Goudreau, P. N., 2000. Two-component signal transduction. Annual Review of Biochemistry. 69**,** 183-215.

Stratford, M., Anslow, P. A., 1996. Comparison of the inhibitory action on *Saccharomyces cerevisiae* of weak-acid preservatives, uncouplers, and medium-chain fatty acids. Fems Microbiology Letters. 142**,** 53-58.

Stromback, L., Lambrix, P., 2005. Representations of molecular pathways: an evaluation of SBML, PSI MI and BioPAX. Bioinformatics. 21**,** 4401-4407.

Sullivan, L., Bennett, G. N., 2006. Proteome analysis and comparison of *Clostridium acetobutylicum* ATCC 824 and Spo0A strain variants. Journal of Industrial Microbiology & Biotechnology. 33**,** 298-308.

Talarico, T. L., Dev, I. K., Dallas, W. S., Ferone, R., Ray, P. H., 1991. Purification and partial characterization of 7,8-dihydro-6-hydroxymethylpterin-pyrophosphokinase and 7,8-dihydropteroate synthase from *Escherichia coli* MC4100. Journal of Bacteriology. 173**,** 7029-7032.

Tang, X., Tan, Y., Zhu, H., Zhao, K., Shen, W., 2009. Microbial Conversion of Glycerol to 1,3-Propanediol by an Engineered Strain of *Escherichia coli*. Applied and Environmental Microbiology. 75**,** 1628-1634.

Thiel, K. A., 2004. Biomanufacturing, from bust to boom ... to bubble? Nature Biotechnology. 22**,** 1365-1372.

Thomas, J. D., Daniel, R. A., Errington, J., Robinson, C., 2001. Export of active green fluorescent protein to the periplasm by the twin-arginine translocase (Tat) pathway in *Escherichia coli*. Molecular Microbiology. 39**,** 47-53.

Thompson, J. L., Hinton, M., 1996. Effect of short-chain fatty acids on the size of enteric bacteria. Letters in Applied Microbiology. 22**,** 408-412.

Tobe, T., Nakanishi, N., Sugimoto, N., 2011. Activation of Motility by Sensing Short-Chain Fatty Acids via Two Steps in a Flagellar Gene Regulatory Cascade in Enterohemorrhagic *Escherichia coli*. Infection and Immunity. 79**,** 1016-1024.

Tomas, C. A., Beamish, J., Papoutsakis, E. T., 2004. Transcriptional analysis of butanol stress and tolerance in *Clostridium acetobutylicum*. Journal of Bacteriology. 186**,** 2006-2018.

Tomas, C. A., Welker, N. E., Papoutsakis, E. T., 2003. Overexpression of *groESL* in *Clostridium acetobutylicum* results in increased solvent production and tolerance, prolonged metabolism, and changes in the cell's transcriptional program. Applied and Environmental Microbiology. 69**,** 4951-4965.

Tomita, M., Hashimoto, K., Takahashi, K., Shimizu, T. S., Matsuzaki, Y., Miyoshi, F., Saito, K., Tanida, S., Yugi, K., Venter, J. C., Hutchison, C. A., 1999. E-CELL: software environment for whole-cell simulation. Bioinformatics. 15**,** 72-84.

Tsuchida, T., Momose, H., 1986. Improvement of an L-leucine-producing mutant of *Brevibacterium lactofermentum* 2256 by genetically desensitizing it to α -acetohydroxy acid synthetase. Applied and Environmental Microbiology. 51**,** 1024-1027.

Tucker, D. L., Tucker, N., Conway, T., 2002. Gene expression profiling of the pH response in *Escherichia coli*. Journal of Bacteriology. 184**,** 6551-6558.

Tummala, S. B., Junne, S. G., Paredes, C. J., Papoutsakis, E. T., 2003. Transcriptional analysis of product-concentration driven changes in cellular programs of recombinant *Clostridium acetobutylicum* strains. Biotechnology and Bioengineering. 84**,** 842-854.

van den Berg, B., 2010. Going Forward Laterally: Transmembrane Passage of Hydrophobic Molecules through Protein Channel Walls. Chembiochem. 11**,** 1339-1343.

Van den Bulcke, T., Lemmens, K., Van de Peer, Y., Marchal, K., 2006. Inferring transcriptional networks by mining 'Omics' data. Current Bioinformatics. 1**,** 301-313.

van Ommen, B., 2004. Nutrigenomics: Exploiting systems biology in the nutrition and health arenas. Nutrition. 20**,** 4-8.

Vemuri, G. N., Aristidou, A. A., 2005. Metabolic engineering in the -omics era: Elucidating and modulating regulatory networks. Microbiology and Molecular Biology Reviews. 69**,** 197-+.

Vendeville, A., Winzer, K., Heurlier, K., Tang, C. M., Hardie, K. R., 2005. Making 'sense' of metabolism: Autoinducer-2, LuxS and pathogenic bacteria. Nature Reviews Microbiology. 3**,** 383-396.

Vergara, M., Becerra, S., Berrios, J., Osses, N., Reyes, J., Rodriguez-Moya, M., Gonzalez, R., Altamirano, C., 2014. Differential Effect of Culture Temperature and Specific Growth Rate on CHO Cell Behavior in Chemostat Culture. Plos One. 9.

Vertes, A. A., Inui, M., Yukawa, H., 2005. Manipulating corynebacteria, from individual genes to chromosomes. Applied and Environmental Microbiology. 71**,** 7633-7642.

Vescovi, E. G., Soncini, F. C., Groisman, E. A., 1996. Mg^{2+} as an extracellular signal: Environmental regulation of *Salmonella* virulence. Cell. 84**,** 165-174.

Wang, F., Zhong, N.-Q., Gao, P., Wang, G.-L., Wang, H.-Y., Xia, G.-X., 2008. SsTypA1, a chloroplast-specific TypA/BipA-type GTPase from the halophytic plant *Suaeda salsa*, plays a role in oxidative stress tolerance. Plant Cell and Environment. 31**,** 982-994.

Wang, R. S., Wang, Y., Zhang, X. S., Chen, L., 2007. Inferring transcriptional regulatory networks from high-throughput data. Bioinformatics. 23**,** 3056-3064.

Wang, X., Miller, E. N., Yomano, L. P., Shanmugam, K. T., Ingram, L. O., 2012. Increased Furan Tolerance in *Escherichia coli* Due to a Cryptic ucpA Gene. Applied and Environmental Microbiology. 78**,** 2452-2455.

Watt, S. A., Patschkowski, T., Kalinowski, J., Niehaus, K., 2003. Qualitative and quantitative proteomics by two-dimensional gel electrophoresis, peptide mass fingerprint and a chemically-coded affinity tag (CCAT). Journal of Biotechnology. 106**,** 287-300.

Wei, P., Li, Z., He, P., Lin, Y., Jiang, N., 2008. Genome shuffling in the ethanologenic yeast *Candida krusei* to improve acetic acid tolerance. Biotechnology and Applied Biochemistry. 49**,** 113-120.

Westergaard, S. L., Oliveira, A. P., Bro, C., Olsson, L., Nielsen, J., 2007. A systems biology approach to study glucose repression in the yeast Saccharomyces cerevisiae. Biotechnology and Bioengineering. 96**,** 134-145.

Wilkins, M. R., Gasteiger, E., Bairoch, A., Sanchez, J. C., Williams, K. L., Appel, R. D., Hochstrasser, D. F., 1999. Protein identification and analysis tools in the ExPASy server. 2-D Proteome Analysis Protocols. 112**,** 531-552.

Wilks, J. C., Slonczewski, J. L., 2007. pH of the cytoplasm and periplasm of *Escherichia coli:* Rapid measurement by green fluorescent protein fluorimetry. Journal of Bacteriology. 189**,** 5601-5607.

Williams, T. I., Combs, J. C., Lynn, B. C., Strobel, H. J., 2007. Proteomic profile changes in membranes of ethanol-tolerant *Clostridium thermocellum*. Applied Microbiology and Biotechnology. 74**,** 422-432.

Wurm, F. M., 2004. Production of recombinant protein therapeutics in cultivated mammalian cells. Nature Biotechnology. 22**,** 1393-1398.

Xavier, K. B., Bassler, B. L., 2005. Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in *Escherichia coli*. Journal of Bacteriology. 187**,** 238-248.

Yamada, M., Makino, K., Amemura, M., Shinagawa, H., Nakata, A., 1989. Regulation of the phosphate regulon of *Escherichia coli* - Analysis of mutant *phoB* and *phoR* genes causing different phenotypes. Journal of Bacteriology. 171**,** 5601-5606.

Yamamoto, K., Ogasawara, H., Fujita, N., Utsumi, R., Ishihama, A., 2002. Novel mode of transcription regulation of divergently overlapping promoters by PhoP, the regulator of two-component system sensing external magnesium availability. Molecular Microbiology. 45**,** 423-438.

Yang, S. H., Tschaplinski, T. J., Engle, N. L., Carroll, S. L., Martin, S. L., Davison, B. H., Palumbo, A. V., Rodriguez, M., Brown, S. D., 2009. Transcriptomic and metabolomic profiling of *Zymomonas mobilis* during aerobic and anaerobic fermentations. BMC Genomics. 10.

Yazdani, S. S., Gonzalez, R., 2008. Engineering *Escherichia coli* for the efficient conversion of glycerol to ethanol and co-products. Metabolic Engineering. 10**,** 340-351.

Yee, J. C., Gatti, M. d. L., Philp, R. J., Yap, M., Hu, W.-S., 2008. Genomic and proteomic exploration of CHO and hybridoma cells under sodium butyrate treatment. Biotechnology and Bioengineering. 99**,** 1186-1204.

Yoshida, T., Qin, L., Egger, L. A., Inouye, M., 2006. Transcription regulation of *ompF* and *ompC* by a single transcription factor, OmpR. Journal of Biological Chemistry. 281**,** 17114-17123.

Yu, X., Liu, T., Zhu, F., Khosla, C., 2011. *In vitro* reconstitution and steady-state analysis of the fatty acid synthase from *Escherichia coli*. Proceedings of the National Academy of Sciences of the United States of America. 108**,** 18643-18648.

Zha, W. J., Rubin-Pitel, S. B., Shao, Z. Y., Zhao, H. M., 2009. Improving cellular malonyl-CoA level in *Escherichia coli* via metabolic engineering. Metabolic Engineering. 11**,** 192-198.

Zhang, F., Ouellet, M., Batth, T. S., Adams, P. D., Petzold, C. J., Mukhopadhyay, A., Keasling, J. D., 2012. Enhancing fatty acid production by the expression of the regulatory transcription factor FadR. Metabolic Engineering. 14**,** 653-660.

Zhang, S. L., Ye, B. C., Chu, J., Zhuang, Y. P., Guo, M. J., 2006. From multi-scale methodology to systems biology: to integrate strain improvement and fermentation optimization. Journal of Chemical Technology and Biotechnology. 81**,** 734-745.

Zhang, X. J., Li, M., Agrawal, A., San, K. Y., 2011. Efficient free fatty acid production in *Escherichia coli* using plant acyl-ACP thioesterases. Metabolic Engineering. 13**,** 713-722.

Zhu, Y., Li, J., Tan, M., Liu, L., Jiang, L., Sun, J., Lee, P., Du, G., Chen, J., 2010. Optimization and scale-up of propionic acid production by propionic acid-tolerant *Propionibacterium acidipropionici* with glycerol as the carbon source. Bioresource Technology. 101**,** 8902-8906.

Appendix

Table A1. Strains and Plasmids used in this study.

Strain/Plasmid	Description/Genotype	Source
MG1655	F- λ- *ilvG- rfb*-50 *rph*-1	(Kang et al., 2004)
BW25113	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), *λ-*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*, pKD46	(Baba et al., 2006)
BW25113 Δ*ompF*	BW25113 Δ*ompF*::FRT-kan-FRT	(Baba et al., 2006)
BW25113 Δ*sodA*	BW25113 Δ*sodA*::FRT-kan-FRT	(Baba et al., 2006)
BW25113 Δ*sodB*	BW25113 Δ*sodB*::FRT-kan-FRT	(Baba et al., 2006)
BW25113 Δ*fliC*	BW25113 Δ*fliC*::FRT-kan-FRT	(Baba et al., 2006)
BW25113 Δ*pflB*	BW25113 Δ*pflB*::FRT-kan-FRT	(Baba et al., 2006)
BW25113 Δ*ppiB*	BW25113 Δ*ppiB*::FRT-kan-FRT	(Baba et al., 2006)
BW25113 Δ*lpdA*	BW25113 Δ*lpdA*::FRT-kan-FRT	(Baba et al., 2006)
BW25113 Δ*ptsH*	BW25113 Δ*ptsH*::FRT-kan-FRT	(Baba et al., 2006)
BW25113 Δ*eno*	BW25113 Δ*eno*::FRT-kan-FRT	(Baba et al., 2006)
BW25113 Δ*rpsA*	BW25113 Δ*rpsA*::FRT-kan-FRT	(Baba et al., 2006)
BW25113 Δ*trxA*	BW25113 Δ*trxA*::FRT-kan-FRT	(Baba et al., 2006)
BW25113 Δ*poxB*	BW25113 Δ*poxB*::FRT-kan-FRT	(Baba et al., 2006)
BW25113 Δ*ompF* Δ*pflB*	BW25113 Δ*ompF*::FRT Δ*pflB*::FRT-kan-FRT	This study
BW25113 Δ*adhE*	BW25113 Δ*adhE*::FRT-kan-FRT	(Baba et al., 2006)
BW25113 Δ*gldA*	BW25113 Δ*gldA*::FRT-kan-FRT	(Baba et al., 2006)
BW25113 Δ*adhE* Δ*gldA*	BW25113 Δ*adhE*::FRT Δ*gldA*::FRT-kan-FRT	This study
AG1	*recA1 endA1 gyrA96 thi-1 hsdR17(rK- mK+) supE44 relA1*	(Kitagawa et al., 2005)
pCA24N, -gfp	Cm^r; $lacI^q$ pCA24N	(Kitagawa et al., 2005)
pCA24N *ompF*	*E. coli ompF* gene under control of pT5/lac	(Kitagawa et al.,

	promoter	2005)
pCA24N *sodA*	*E. coli sodA* gene under control of pT5/lac promoter	(Kitagawa et al., 2005)
pCA24N *sodB*	*E. coli sodB* gene under control of pT5/lac promoter	(Kitagawa et al., 2005)
pCA24N *fliC*	*E. coli fliC* gene under control of pT5/lac promoter	(Kitagawa et al., 2005)
pCA24N *pflB*	*E. coli pflB* gene under control of pT5/lac promoter	(Kitagawa et al., 2005)
pCA24N *ppiB*	*E. coli ppiB* gene under control of pT5/lac promoter	(Kitagawa et al., 2005)
pCA24N *lpdA*	*E. coli lpdA* gene under control of pT5/lac promoter	(Kitagawa et al., 2005)
pCA24N *ptsH*	*E. coli ptsH* gene under control of pT5/lac promoter	(Kitagawa et al., 2005)
pCA24N *eno*	*E. coli eno* gene under control of pT5/lac promoter	(Kitagawa et al., 2005)
pCA24N *rpsA*	*E. coli rpsA* gene under control of pT5/lac promoter	(Kitagawa et al., 2005)
pCA24N *trxA*	*E. coli trxA* gene under control of pT5/lac promoter	(Kitagawa et al., 2005)
pCA24N *poxB*	*E. coli poxB* gene under control of pT5/lac promoter	(Kitagawa et al., 2005)
pTrcHis2A (pTH_A)	pTrcHis2A (pBR322-derived), oriR pMB1, *lacI*^{q}, *bla*	Invitrogen (Carlsbad, CA)
pTH_A *ompF*	*E. coli ompF* gene under control of P*trc* promoter	This study
pTH_A *pflB*	*E. coli pflB* gene under control of P*trc* promoter	This study
pTH_A *poxB*	*E. coli poxB* gene under control of P*trc* promoter	This study
pTH_A *sodB*	*E. coli sodB* gene under control of P*trc* promoter	This study
pTL_K	pTrcHis2A containing p15A oriR from pACYC184, Kan^R	(Blankschien et al., 2010), This study
pTL_K *ompF*	*E. coli ompF* gene under control of P*trc* promoter	This study
pBad24-TorA-GFPmut3* (pJDT1)	Amp^R, *araC*, P_{BAD}, pBR322 *ori*, *Nco*I-*Hind*III TorA::GFP	(Thomas et al., 2001)

Table A2. Primers used for plasmid construction

Plasmid Construction	Primer Sequences and Restriction Enzymes
pTH_A-*ompF*	5'-GAGGAATAAACCATGATGAAGCGCAATATTCTGG-3' 5'-CGTTTACCAGTTCTAATAGCACACCGAATTCGAAGCTTGGG-3' (pTH_A, NcoI/EcoRI)
pTH_A-*pflB*	5'-GAGGAATAAACCATGTCCGAGCTTAATGAAAAGTTA-3' 5'-CCCAAGCTTCGAATTCGCGGCCGCTTACATAGATTGAGTGAAGGTACGAGT-3' (pTH_A, NcoI/EcoRI)
pTH_A-*poxB*	5'-GAGGAATAAACCATGAAACAAACGGTTGCAG-3' 5'-CCCAAGCTTCGAATTCGCGGCCGCAATGCCTTACCTTAGCCAGTTT-3' (pTH_A, NcoI/EcoRI)
pTH_A-*sodB*	5'-GAGGAATAAACCATGTCATTCGAATTACCTGCACTAC-3' 5'-CCCAAGCTTCGAATTCGCGGCCGCCAGTTATTATGCAGCGAGATTTTT-3' (pTH_A, NcoI/EcoRI)
pTL-*ompF*	5'-GAGGAATAAACCATGATGAAGCGCAATATTCTGG-3' 5'-CGTTTACCAGTTCTAATAGCACACCGAATTCGAAGCTTGGG-3' (pTL, NcoI/EcoRI)
pTL_K-replacing amp with kan marker	5'-TGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGATTGAACAAGATGGATTGC-3' 5'-TGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTCAGAAGAACTCGTCAAGAAGG-3'
pTL_K-site directed mutagenesis	5'-GATCTCGTCGTGACCCACGGCGATGCCTGCTTGC-3' 5'-GCAAGCAGGCATCGCCGTGGGTCACGACGAGATC-3'